

Cloning of Actin Genes
from a Genomic Library of the Newt, Notophthalmus viridescens

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ABSTRACT:

The regenerating urodele limb is a useful model system in which to study, *in vivo*, the controls of cell proliferation and differentiation. Techniques are available which enable one to experimentally manipulate mitogenic influences upon the blastema, as well the morphogenesis of the regenerating limb. Although classical regeneration studies have generated a wealth of knowledge concerning tissue interactions, little is known about the process at the level of gene expression.

The aim of this project was to clone potentially developmentally regulated genes from a newt genomic library for use in future studies of gene expression during limb regeneration. We decided to clone the cytoskeletal actin gene for the following reasons: 1. its expression reflects the proliferative and differentiative states of cells in other systems 2. the high copy number of cytoplasmic actin pseudogenes in other vertebrates and the high degree of evolutionary sequence conservation among actin genes increased the chance of cloning one of the newt cytoplasmic actin genes. 3. Preliminary experiments indicated that a newt actin could probably be identified using an available chick β -actin gene for a molecular probe.

Two independent recombinant phage clones, containing actin homologous inserts, were isolated from a newt genomic library by hybridization with the chick actin probe.

Restriction mapping identified actin homologous sequences within the newt DNA inserts which were subcloned into the plasmid pTZ19R. The recombinant plasmids were transformed into the Escherichia coli strain, DH5 α . Detailed restriction maps were produced of the 5.7Kb and 3.1Kb newt DNA inserts in the plasmids, designated pTNA1 and pTNA2. The short (<1.3 Kb) length of the actin homologous sequence in pTNA2 indicated that it was possibly a reverse transcript pseudogene. Problems associated with molecular cloning of DNA sequences from N. viridescens are discussed with respect to the large genome size and abundant highly repetitive DNA sequences.

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TABLE OF CONTENTS:

	PAGE
ABSTRACT	2
ACKNOWLEDGEMENTS	4
TABLE OF CONTENTS	5
LIST OF ILLUSTRATIONS	7
LIST OF TABLES	9
INTRODUCTION	10
LITERATURE REVIEW	
The Regenerating Urodele Limb as an Experimental System	13
The Urodele Genomic Structure	21
The Actin Proteins	24
The Actin Gene Family	34
MATERIALS AND METHODS	
Chemicals, Enzymes and Bacterial Strains	43
Solutions	50
The Genomic Library and Molecular Probes	50
Purification of Genomic DNA	51
EMBL3 Bacteriophage DNA Purification	51
Plasmid DNA Purification	55
Purification of <u>E. coli</u> Carrier DNA	56
Dot Blot	56
Agarose Gel Electrophoresis	57
Southern Blot	57
Plaque Lift	58
Radioactive Labelling of DNA for Probes	58
Hybridization	59
Probe Removal	61

Autoradiography	61
Recombinant DNA Techniques	61
Choice of Plasmid Vector	62
Bacterial Transformation	65
RESULTS	
Dot Blot	67
Southern Blots	70
Library Screening	76
Restriction Site Mapping of EMBL3 Clones	86
Plasmid Cloning	98
Restriction Site Mapping of Plasmid Clones	101
DISCUSSION	117
SUMMARY	128
REFERENCES	129
APPENDIX I	
a. Calculation of the number of EMBL3 phage needed for a complete <u>N. viridescens</u> genomic library.	136
b. Calculation of the actin gene copy number in the newt genome.	137
APPENDIX II	
Diagram of the Southern Blot Procedure	138
APPENDIX III	
a. DNA Hybridization Stringency	141
b. DNA Hybridization Rate	142

LIST OF ILLUSTRATIONS:

FIGURE	PAGE
1. Stages in the Regeneration of a newt limb.	15
2. The polar coordinate model.	18
3. Evolutionary relationships of the actin proteins.	26
4. Molecular structure of the actin protein.	29
5. β -actin gene structure.	37
6. Cloning sequences from an EMBL3 genomic library.	44
7. Subcloning DNA fragments into plasmids.	46
8. Plasmids used as probes.	52
9. The pTZ19R plasmid.	63
10. Dot blot of genomic DNA from several species probed with c-fos, c-myc, and β -actin.	68
11. Southern blot of genomic DNA from several species probed with c-fos and β -actin.	71
12. Southern blot of genomic DNA from several species probed with β -actin.	74
13. Southern blot of newt library DNA probed with β -actin.	77
14. Southern blot of newt library DNA probed with c-fos.	79
15. Examples of primary plaque lifts probed with β -actin.	81
16. Secondary screening of the library.	84
17. Restriction fragment and Southern analysis of phage 11A.	87
18. Further restriction fragment and Southern analysis of phage 11A.	89
19. Restriction map of EMBL3 clone 11A.	92
20. Rescreening of the genomic library and restriction fragment and Southern analysis of phage clone 16AA.	94
21. Restriction map of EMBL3 clone 16AA.	96

22.	Selection of plasmid subclone pTNA1.	99
23.	Restriction fragment analysis of pTNA1.	102
24.	Restriction fragment and Southern analysis of pTNA1.	104
25.	Restriction map of pTNA1.	106
26.	Selection of plasmid subclone pTNA2.	108
27.	Restriction fragment and Southern analysis of pTNA2 and pTNA1.	110
28.	Restriction fragment and Southern analysis of pTNA2.	112
29.	Restriction map of pTNA2.	114
30.	EMBL3 phage replication.	122
31.	Southern Blot Procedure.	139

LIST OF TABLES:

TABLE	PAGE
1. Actin-binding Proteins	31
2. Bacterial Strains Used	48

INTRODUCTION

Proliferation and differentiation are fundamental processes in cell biology. Their control provides the basis for pattern formation during embryogenesis and tissue regeneration and for the maintenance of organisms through replacement of specific cells and wound healing. Inappropriate proliferation and or differentiation of cells leads to a variety of diseases including cancer. The ability to control these processes in cells would allow improvements in the treatment of such diseases and many other medical problems, from healing injuries to the synthesis of pharmaceutical products.

There has been substantial progress, particularly recently, towards understanding the processes of cell proliferation and differentiation. A very fruitful approach has been to study changes in gene expression which cause or accompany changes in the differentiative or proliferative state of cells. Cell culture or embryonic systems are commonly used for such experiments. The regenerating urodele limb is a particularly good model system in which to study proliferation and differentiation, but as yet not many studies have been done at the molecular level. During limb regeneration, there are opportunities to experimentally intervene as cells go through stages of dedifferentiation, proliferation, and redifferentiation. The proliferation of blastema cells is strictly dependent on innervation, which

can be manipulated by removal or redirection of nerves.⁵ Pattern formation can be altered by surgical procedures¹⁴ or injection of morphogenic substances such as retinoic acid.¹¹

As a starting point to the study of the molecular controls of proliferation and differentiation during limb regeneration, I have attempted to clone a developmentally regulated gene from the newt genome. Three possible genes were chosen for the project: two oncogenes c-fos and c-myc, and β -actin which encodes a cytoskeletal protein. In other systems, changes in the expression of these genes have been found to accompany the differentiation,⁶⁹ response to mitogens,⁷⁰ and neoplastic transformation of cells.⁷⁷ It would therefore be useful to follow the expression of these genes during limb regeneration. Cloning of the newt genes is necessary to produce the molecular probes used in such a study. The use of genes from other species as probes can make detection of mRNA difficult, due to the sequence divergence of the probes.

The DNA sequences of c-fos, c-myc, and β -actin are evolutionarily conserved,^{32,70} increasing the likelihood of cloning these genes from the newt. Preliminary experiments indicated that β -actin would present the least difficulties, and so it was decided to screen a Notophthalmus viridescens genomic library for actin related sequences. In addition to facilitating studies of actin gene expression, cloning of the newt actin gene and characterization of its structure would yield some information about the relation of newt actin genes to other cloned actin genes, and shed some light on the

organization of the unusually large newt genome. Questions such as the number of newt actin genes and their sequence divergence from other cloned actin genes could be addressed.

LITERATURE REVIEW:

The Regenerating Urodele Limb as an Experimental System

The regenerating urodele limb is a unique model system in which to study cells as they progress through several changes in their state of proliferation and differentiation. After amputation and wound healing, stump tissues dedifferentiate and accumulate under the wound epithelium as a rapidly proliferating mass of cells called the blastema. As the regenerate elongates, cells begin to redifferentiate in a proximal to distal direction until a morphologically complete limb is formed. (see Fig.1)

THE REGENERATION PROCESS

Classical experiments have provided much information about the process of regeneration. The cells in the blastema are derived from a variety of tissue types which actually dedifferentiate to form cells with embryonic morphology.² Blastema cells can redifferentiate into different tissue types than those from which they originated. For example, it was shown by Weiss that a limb with the humerus removed will regenerate normal skeletal structures distal to an upper arm amputation.³

The redifferentiation to form limb structures does not proceed unless a certain critical mass of blastema cells is accumulated.³ If cell division is inhibited prior to this, there is no differentiation. However, if mitosis is

Fig.1

Depicts stages in the regeneration of a newt limb after amputation through the mid-humerus. AMP- amputation, WH- wound healing, EDD- early dedifferentiation, LDD- late dedifferentiation, MEB- mid-early bud, EB- early blastema, MB- mid blastema, LB- late blastema, Pal- pallet, ED- early digit, MD- mid digit, LD- late digit. (From Iten¹)



Amp



WH



EDD



LDD



MEB



EB



MB



LB



LB



Pal



ED



ED



MD



LD

repressed once a large enough blastema has been formed, a smaller but morphologically complete limb is formed.^{4,5}

A major proliferative influence on the blastema is provided by innervation. As long as 150 years ago it was recognized that denervation prevented regeneration.⁶ Since that time many researchers have searched for neurotrophic substances. Several proteins have been purified from neural tissue that are mitogenic for blastema cells. Some examples are fibroblast growth factor(FGF),⁷ glial growth factor(GGF),⁸ substance P,⁹ and chick brain growth factor(CBGF).¹⁰ The role of these mitogens, in vivo, is not certain though.

PATTERN FORMATION

The problem of how positional information is established in the blastema has also been approached. Studies of the regenerating urodele limb have led to the polar coordinate(PC) model of positional information possessed by cells.¹¹ This model proposes that cells in the limb have information about their position along the proximo-distal axis and around the limb circumference.(see Fig.2a) It also proposes the important feature that positional value discontinuity is solved by intercalation and distalization. When cells from normally non-adjacent positions contact each other, cell division is stimulated to produce cells with the intermediate positional values that are missing. The regenerating tissue always becomes distalized compared to preexisting cells.

This theory attempts to explain the results of several

Fig.2a,b,c

Fig.2a. The polar coordinate model: cells contain positional information about their location along the proximo-distal axis and around the limb circumference.

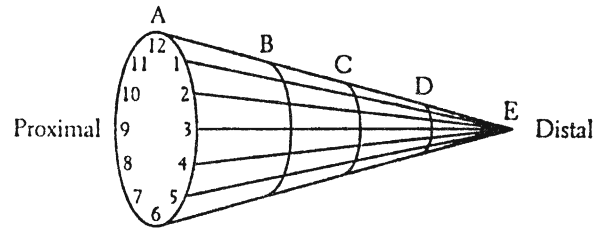
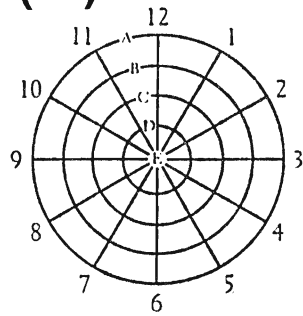
Fig.2b. Distalization occurs during regeneration of a reversed polarity limb.

Fig.2c. Circumferential intercalation occurs, producing supernumerary limbs, when a blastema is rotated so that positional information of adjacent cells is mismatched. (A-anterior, P-posterior, D-dorsal, V-ventral)

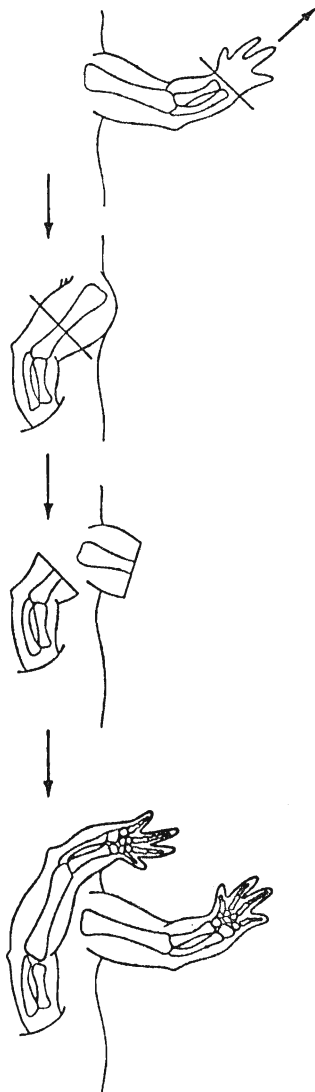
Fig.2d. Intercalation in the proximo-distal axis occurs in response to the grafting of a wrist level blastema onto a shoulder stump.

These figures are from Walbot.¹³

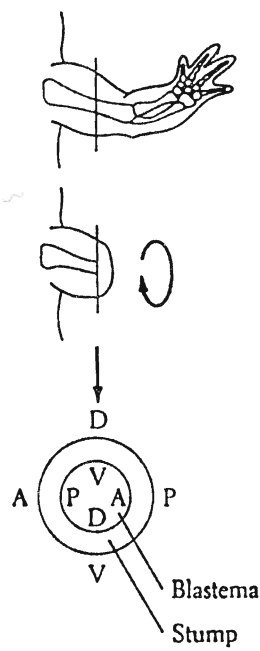
(A)



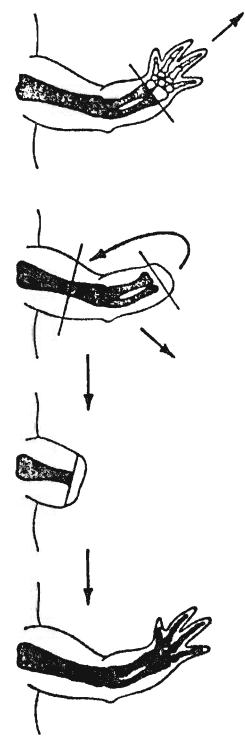
(B)



(C)



(D)



classical blastema transplantation experiments.

Distalization was demonstrated elegantly by Butler¹² with the creation of a reversed polarity limb by removing the hand of a urodele forelimb and allowing the wrist to heal into a slit in the flank. When the bent limb was then amputated through the humerus, the limb regenerated the distal part, despite the reversed polarity, so that the humerus was flanked by two sets of radius and ulna.(Fig.2b)

Circumferential intercalation is exemplified by the development of supernumerary limbs following rotation of the blastema.¹⁴ This places ventral and anterior tissues of the blastema next to dorsal and posterior tissues of the stump and visa versa. The resulting intercalary proliferation establishes an extra limb regenerate on each side of the original. The extra limbs are of opposite handedness.(Fig.2c)

Intercalation in the proximo-distal axis occurs when the blastema from a wrist level amputation is grafted onto a shoulder amputation.¹⁵ Cells from the shoulder stump proliferate until humerus, radius and ulna have been regenerated to replace tissues intermediate to the wrist and shoulder. The hand then regenerates.(Fig.2d)

Recently, numerous studies have investigated the effects of retinoids(vitamin A and analogues) on pattern formation.¹¹ These chemicals cause the regeneration of extra elements in the proximo-distal axis of the newt limb. Examination of treated blastemas revealed inhibition of cell division, breakdown of the stump cartilage, and aggregation of the

mesenchymal cells which are normally evenly dispersed.¹¹

There are very few studies on gene expression at the mRNA level in the regenerating amphibian limb. Brockes(personal communication) has isolated a newt myosin gene and followed its expression during dedifferentiation and redifferentiation of muscle tissue. Investigation of genes that are regulated during limb regeneration would lead to a better understanding of regeneration and the underlying processes of cell proliferation and differentiation. In particular, it would be interesting to see how gene expression is affected by experimental manipulations of the type described above.

The Urodele Genomic Structure

LAMPBRUSH CHROMOSOMES

Investigations of the genetics of urodeles have long been fostered by interest in the oocyte lamp brush chromosomes, which can be observed with a light microscope,¹⁶ and by the unusually large size of the urodele genome.¹⁶

Lampbrush chromosomes occur in the oocyte when threads are thrown out transverse to the main axes of the chromosomes. These loops are thought to represent active sections of the chromosome which have become despiralized.¹⁷ This makes it convenient to study gene activation by direct observation or by in situ hybridization of genetic probes to nascent mRNA on the loops. This technique has been used to identify genes transcribed during oogenesis and map them to specific loci.^{16,18}

GENOME STRUCTURE

Some urodele genomes are as much as 25 times the size of mammalian genomes.^{19,20} N. viridescens has one of the highest known DNA values, 45 pg per haploid genome.²¹ Much of this DNA is repetitive. Generally, as the genome size of amphibians increases, so does the proportion of repetitive DNA in the genome.¹⁹ For amphibians with genomes approximately the same size as N. viridescens, the highly repetitive fraction makes up from 40 to 70 percent.¹⁹ It is not known whether this repetitive DNA has a function or by what mechanism it has evolved. Some of the repetitive DNA is

classified as satellite DNA; tandemly repeated sequences, less than 1000 bp in length, present in more than 10^5 copies per haploid genome.²²

SATELLITE DNA

Satellite DNA sequences were among the first DNA fragments cloned from newts.¹⁸ Two such satellite sequences in *N. viridescens*, which make up 0.7% of the genome,²² are transcribed.¹⁸ One of these, the satellite-2 sequence, produces unusually stable transcripts which possess self-cleaving catalytic activity.²³ Unlike other satellite sequences, located in heterochromatic regions of the chromosomes, satellite-2 is dispersed uniformly throughout the genome.²³

The function of satellite sequences, and particularly their transcripts, is a mystery. In other organisms, satellite DNA is generally limited to heterochromatic regions, and is ascribed a role in chromosome pairing and segregation during meiosis, structural rearrangement of chromosomes, and regulation of gene expression.²⁴ Macgregor and Sessions²² suggest that the adaptive significance of satellite DNA may lie in its potential effect on the rate of cell proliferation and therefore the growth rate of the organism.

The large size of the urodele genome leads to several problems for molecular genetic investigations. For example when screening genomic libraries, the large size requires that many more recombinant phage be screened to find a single copy gene. Compared to mammals, a complete *N. viridescens*

library would contain 15 times as many clones. The large size also makes Southern analyses difficult. Using the same amount of DNA for analysis, sensitivity of detecting single copy genes is 15 times less for newts than for mammals.

Extensive repetitive DNA also presents a problem. DNA propagated in E. coli may become rearranged due to host recombination systems. Repetitive DNA exacerbates this problem as observed by others cloning urodele genomic DNA.¹⁶

The Actin Proteins

THE ACTIN FAMILY

Actin is the major structural protein and one of the most abundant proteins in animal cells. It usually exists as a family of isomorphs, with the number of different isomorphs in the family varying between species. The actin protein is very highly conserved between isomorphs and species. For example, there are fewer than 22 amino acid changes out of 375 between the most divergent isomorphs in the mammalian actin family,²⁶ and only one amino acid change between the β -actins of human and chicken.²⁷

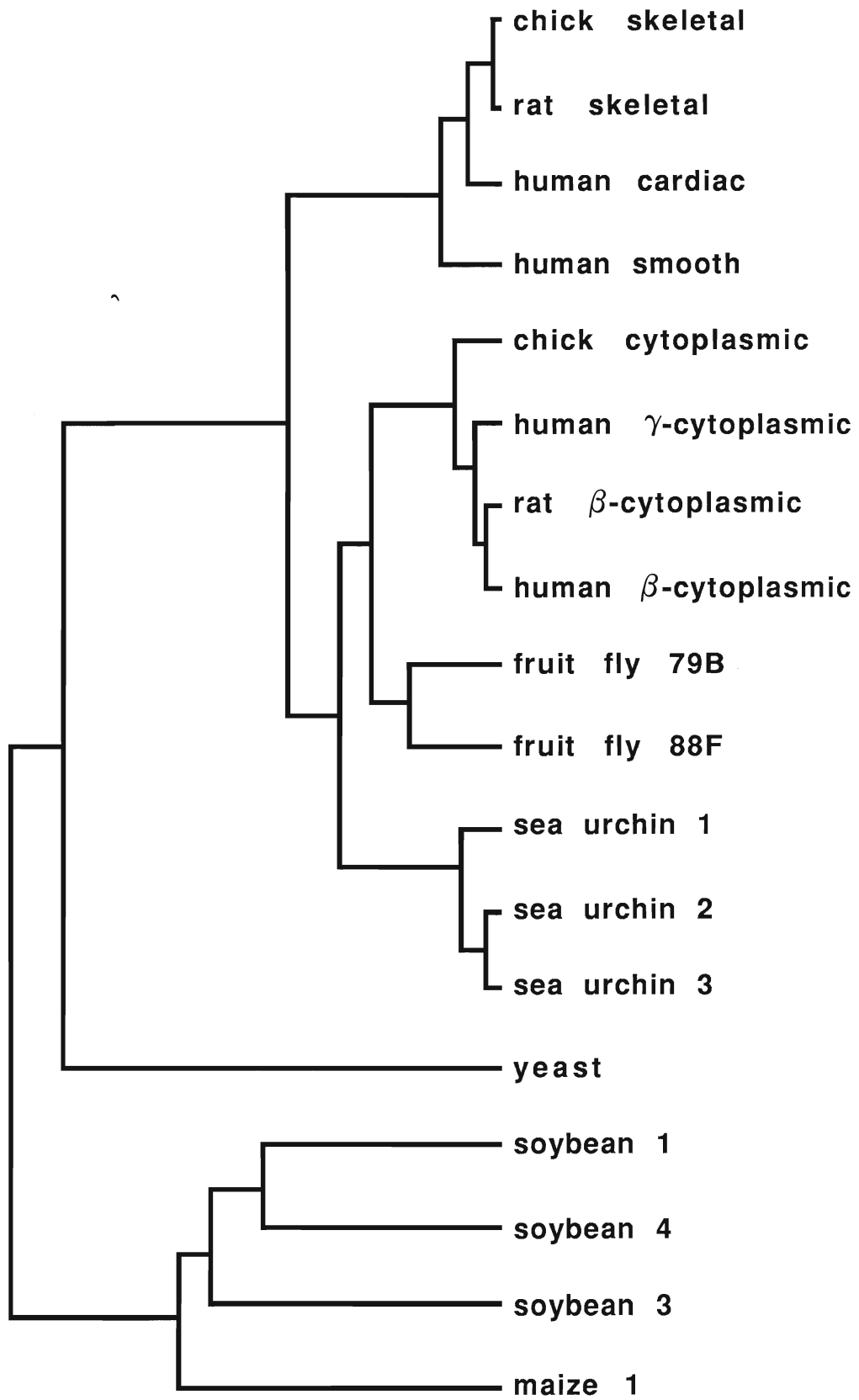
In warm blooded vertebrates, there are four muscle actins; skeletal, cardiac, aortic smooth, stomach smooth; and two cytoskeletal actins, β and γ .^{26,28} In amphibians there are at least five different cytoskeletal actins, individual species having from one to three.²⁹ They are classified as types 1-8 based on the three acidic amino-terminal amino acids.²⁹ Three cytoskeletal actins are found in the newt Triturus cristatus.²⁹ Actins have been studied in many eukaryotes, from Saccharomyces cerevisiae to Dictyostelium sp.³⁰ and plants.³¹ Fig.3 shows the evolutionary relationships between actin proteins.

PROTEIN STRUCTURE³²

Actin takes two forms: the 42kd monomer, called G-actin and the filamentous polymer F-actin. The actin microfilament is a double stranded helix with 13.5 monomers per turn. It

Fig.3

The evolutionary relationships of various actin proteins, based on differences in their amino acid sequences.
(From Hightower.³²)



is approximately 7nm in width and as long as several um or 1000 monomers.(Fig.4) Polymerization of G-actin to F-actin is a complex equilibrium process. Microfilaments may be assembled and disassembled rapidly in the cell and these processes may occur simultaneously at different ends of the same filament, called treadmilling. G-actin is stabilized by Ca^{++} or by binding to other proteins such as profilin. The polymerization process is stimulated by ATP hydrolysis and inhibited by cytochalasins. Microfilaments have polarity, with the polymerization rate differing between the two ends. F-actin is stabilized by phalloidin, a toxic alkaloid produced by the fungus Amanita phalloides, and disassembled by the cellular proteins gelsolin and villin. Microfilaments provide cells with structural support and, in combination with myosin, form contractile systems.

THE ROLE OF ACTIN IN CYTOSKELETAL STRUCTURE³³

Actin filaments make up a large portion of the cell's cytoskeleton, with other major structural filaments consisting of mainly tubulin. The actin filaments are cross-linked by other proteins(see Table 1) such as fibrin, α -actinin and filamin to form networks or bundles. The cores of microvilli are bundles of actin filaments aligned with the same polarity. Besides determining cell shape, the cytoskeleton probably plays a role in cell adhesion to substrates. Characteristic actin bundles called stress fibres contact the cell membrane specifically at adhesion plaques. The protein vinculin is thought to function in linking the actin fibres to the membrane.

Fig.4

Molecular structure of the actin protein. The G-actin monomer, having two spherical domains, polymerizes to form F-actin. (From Darnell.⁸⁴)

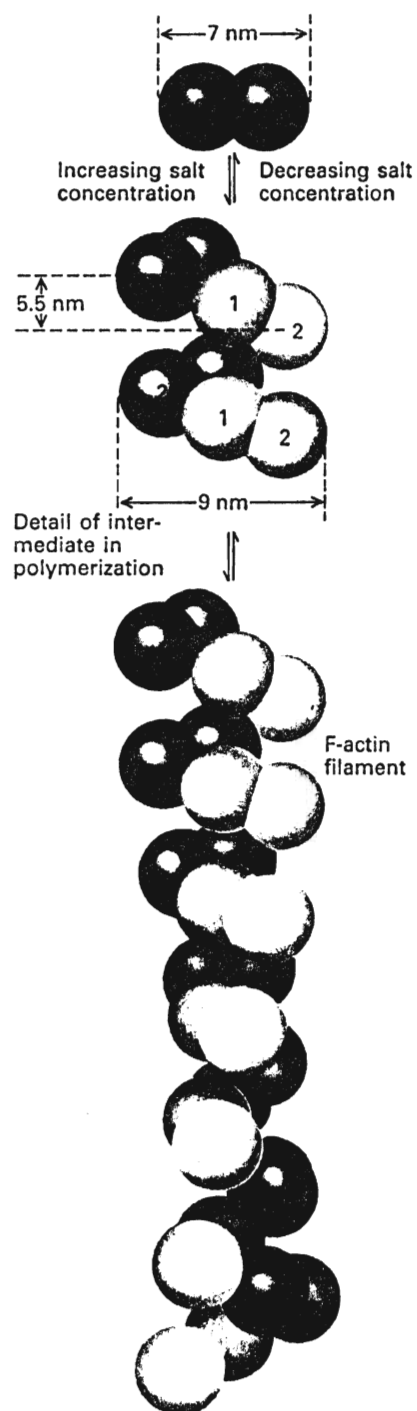


Table 1
Some actin-binding proteins arranged according to
function. (from Weeds³⁴)

	Source	$M_r \times 10^{-3}$	No. of subunits	Ca ²⁺ -sensitivity
Gelation proteins				
Filamin	Muscle	250	2	-
Filamin	Macrophage	270	2	-
Spectrin	Red blood cell	240 (α) 220 (β)	4	-
—	Sea urchin egg	220	-	-
—	<i>Dictyostelium</i>	120	-	-
Gelation and bundling proteins				
α -Actinin	Muscle	105	2	-
α -Actinin	HeLa cells	100	2	+
Actinogelin	Ehrlich ascites	115	-	+
—	<i>Dictyostelium</i>	95	-	+
—	<i>Acanthamoeba</i>	85	-	+
Vinculin	Muscle	130	1	-
Vinculin	HeLa cells	130	1	+
Bundling proteins				
Fascin	Sea urchin egg	58	-	-
Fimbrin	Microvillus	68	1	-
Severing and capping proteins				
β -Actinin	Muscle	34 + 37	1 each	-
Gelsolin	Macrophage	90	1	+
Gelsolin	Platelets	90	1	+
Gelsolin	Plasma	90	1	+
Villin*	Microvillus	95	1	+
Fragmin	<i>Physarum</i>	42	1	+
—	<i>Dictyostelium</i>	40	1	+
Capping protein	<i>Acanthamoeba</i>	28 + 31	1 each	-
Depolymerizing protein	Brain	19	-	-
G-actin stabilizing proteins				
Profilin	Lymphocytes	16	1	-
Profilin	<i>Acanthamoeba</i>	14	1	-

CONTRACTILE FUNCTION³³

Actin contractile systems are of two types, generating movement by either polymerization and depolymerization of filaments or by the sliding of actin against myosin filaments. Actin polymerization and disassembly may change filament length, extending or retracting them. If treadmilling occurs, the filament may change little in length, but it is translocated in the direction of polymerization. Contractile systems execute many cell functions, including locomotion, extension of cell processes and transport of organelles and macromolecules, as in the case of slow axonal transport.

POSSIBLE ROLE OF ACTIN IN CONTROL OF CELL PROLIFERATION AND DIFFERENTIATION

Beyond these structural and contractile functions, it is likely that cytoskeletal actin plays a fundamental role in determining the cellular state of proliferation and differentiation. The arrangement of actin in cells can affect that of neighbouring cells and of daughter cells.³³ In epithelial cells, arrangement of cytoskeletal actin is coordinated to give continuity of filaments within the tissue.³³ In Paramecium sp., cilia arrangement is inherited epigenetically.³³ Such non-genetic inheritance of cytoskeletal characteristics could greatly influence morphogenesis of higher organisms.

It has been long established that changes in arrangement of the cytoskeleton accompany neoplastic transformation of cells. There is a reduction in the number of stress fibres,

cells round up and their interaction with the substrate changes.³³ The actin associated protein vinculin is phosphorylated by the Src oncogene product during transformation.³⁵ Recent evidence indicates that rather than being a consequence of transformation, changes in cytoskeletal actin expression, specifically β -actin, may be an integral part of the progression of cells toward the malignant state.

By treating fibroblasts with chemical mutagens, Leavitt et al isolated a mutant β -actin from neoplastic cells.³⁶ This protein had one amino acid change. Further selection produced cells with additional amino acid changes in β -actin, elevated expression of the mutant protein and increased tumorigenicity.³⁶ Leavitt's group has cloned the gene for this mutant β -actin³⁷ and transfected fibroblasts with it.^{38,39} The resulting cells had altered morphology and the expression of endogenous cytoskeletal actins decreased in a manner consistent with autoregulation of actin expression.^{39,40} The previously nontumorigenic fibroblast cell line, expressing the transfected mutant β -actin, was able to cause tumors in mice.³⁸ These results suggest that expression of mutant β -actin contributes to the neoplastic phenotype.

The Actin Gene Family

As previously mentioned, actin exists in families of protein isomorphs. These are each encoded by a separate gene, resulting in the actin multigene family. In vertebrates, each protein isomorph is the result of a single functional gene.^{41,42} It is likely that some protein isomorphs have not been detected. There appear to be four distinct functional cytoskeletal actin genes in the chicken but only β and γ proteins have been detected.^{27,28}

In addition to the functional genes, many pseudogenes exist for the cytoskeletal types. For example, there are at least 20 human β -actin pseudogenes.⁴³ These differ from the pseudogenes of most other gene families, which are thought to arise by gene duplication.⁴³ The actin pseudogenes are unlinked, being randomly distributed throughout the genome.⁴³ They also lack introns, resembling processed mRNA.⁴³ These are characteristics of 'reverse transcript' or 'processed' pseudogenes, thought to arise from mRNA transcripts.⁴⁴ In many cases the pseudogenes are flanked by repetitive DNA.^{45,46,47}

When the sequence relationships within actin pseudogene families have been carefully studied, they suggest that the pseudogenes have arisen during recent evolution. The human cytoplasmic pseudogenes appear to have arisen during primate evolution⁴⁶ and the mouse pseudogenes have arisen since diversification from rats.⁴¹ Perhaps these pseudogene

families continually and rapidly arise and disappear, possibly originating, as suggested by Minty,⁴¹ from a single processed pseudogene, which is then amplified. The rough correlation between genome size and actin gene number in mammals led Minty to remark that it would be interesting to examine the family in amphibians.⁴¹ Perhaps a greater degree of pseudogene amplification partially accounts for the large amphibian genome size.

STRUCTURE OF THE β -ACTIN GENE

The β -actin genes from many vertebrates have been cloned and sequenced,^{43,48,49} revealing several interesting structural features.(Fig.5b) The transcribed sequence contains 5' and 3' untranslated regions and is separated into five exons. The 5' flanking region, some introns, and the untranslated regions contain sequences with unusually high homology between such distantly related species as human and chicken.²⁶(Fig.5a) The lack of divergence in these non-coding sequences suggests that they have a necessary function.

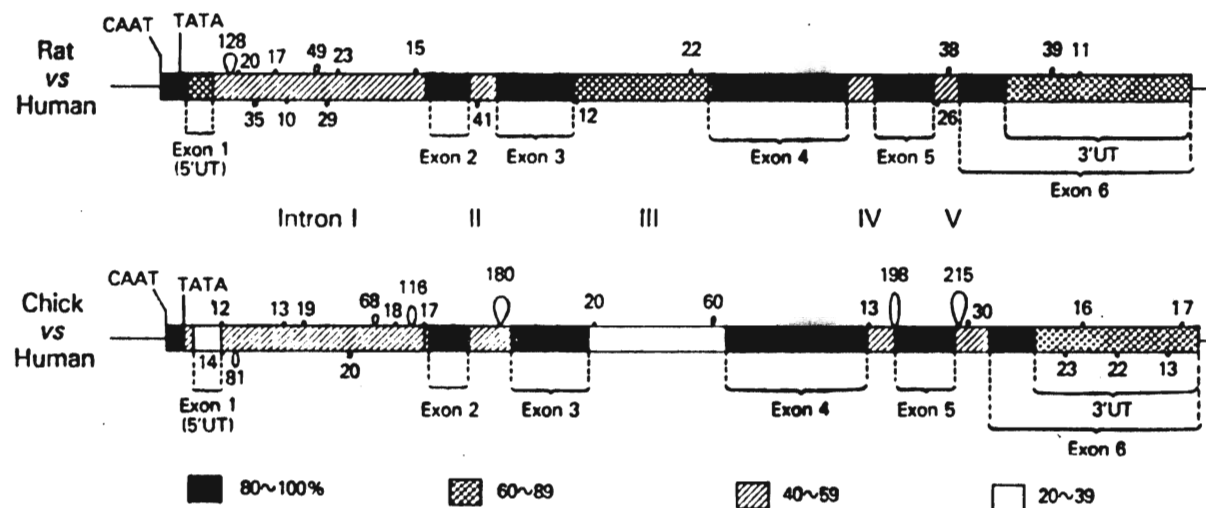
Some of these functions have been partially defined.(Fig.5b) Recent evidence indicates that the 3' end of the β -actin gene is important for tissue specific gene expression.⁵⁰ Within the 5' flanking region there are transcription promotor consensus sequences.⁴³ In this region, as well as in the first intron, there are transcription enhancer elements with the motif CC(A/T)₆GG.⁵¹ These enhancers are homologous to the c-fos protooncogene serum response element(SRE), that is thought to mediate the

Fig.5a,b

Fig.5a. The degree of sequence homology between the human and rat, as well as the human and chicken β -actin gene sequences. Insertions and deletions are shown as loops.
(From Nakajima-Iijima²⁶)

Fig.5b. Structural features of the human β -actin gene.

(A)



(B)



transient transcriptional activation of the c-fos and β -actin genes in response to serum mitogens.^{51,52,53}

The enhancer sequences (among the strongest discovered) alone can activate transcription of heterologous genes when linked to the SV40 virus promotor.⁵⁴ The human β -actin promotor and enhancer regions together have been used to construct expression vectors which direct high level transcription of heterologous genes when transfected into mouse, rat, or human cells.³⁵

DEVELOPMENTAL REGULATION OF ACTIN GENE EXPRESSION

Several groups have investigated the developmental regulation of actin gene expression, and the molecular mechanisms which mediate it. In the early stages of amphibian embryonic development, there is a low, constitutive level of mainly β -cytoskeletal actin mRNA,⁵⁶ and little or no muscle actin gene expression.⁵⁷ By the early neurula stage, Xenopus embryos are morphologically differentiated. Just prior to this stage, in late gastrulation, muscle actin mRNA begins to accumulate but is localized to the somites.^{57,58}

Cytoplasmic factors necessary for the activation of the muscle actin genes are already localized in the precleavage egg, to the dorsal, subequatorial region.⁵⁹ However, the genes remain inactive until late gastrulation,⁶⁰ when contact of the equatorial cells with vegetal cells induces their expression.⁶¹ In vertebrate embryos the skeletal and cardiac types of muscle actin are coexpressed, but later in development the tissue specific expression of the muscle isotypes varies with the developmental stage and

species.^{57,62}

In dividing myoblasts, β -actin is the predominant cytoplasmic form and muscle actins are not present.⁶³ During muscle development, as myoblasts irreversibly fuse to form myotubes, cytoplasmic actins are shed and replaced with muscle actin isoforms.⁶⁴ As myoblasts fuse in culture, or in situ in the embryonic chick, there is a transient 90-fold amplification of the skeletal muscle actin gene, accompanied by a large accumulation of the mRNA.⁶⁵ It is not known whether these amplified copies are all functional or how they are produced and degraded so rapidly.

If β -actin or skeletal actin genes are transfected into myoblasts, the level of β -actin mRNA is correctly down-regulated when the cells are induced to differentiate, but the skeletal actin genes are expressed at low levels even once the muscle cells are differentiated.⁶³ This indicates that unlike β -actin, tissue specific regulation of muscle actin genes requires sequences that are not closely associated with the coding region. Recombinant DNA work has shown that down regulation of β -actin in muscle cells is conferred by the 3' end of the gene or 3' flanking regions,⁵⁰ but sequences 217-416 nucleotides upstream of the transcription start site are necessary for induction of muscle actin in differentiated muscle cells.^{66,67}

REGULATION OF β -ACTIN EXPRESSION BY GROWTH FACTORS

As well as tissue specific regulation, the cytoplasmic actin genes are regulated by factors which alter the proliferation of cells. Changes in gene expression are

coupled to extracellular stimuli by biochemical transduction events. Binding of a growth factor to its receptor is thought to induce conformational changes which internalize the signal. Soluble intracellular second messengers transmit the signal to the nucleus, where changes in gene expression are produced within minutes.

Several genes have been identified which are among the first to be regulated after cells are treated with growth or differentiation factors. The β -actin, c-fos, and c-myc genes all show rapid, transient induction in response to a variety of factors.⁷²

The myc and fos proteins bind to DNA and are thought to regulate other genes.³⁵ They belong to the protooncogene family of genes which have the potential to transform cells so that they become tumorigenic. This property was originally used to identify transforming viral oncogenes. It was subsequently learned that these viral oncogenes were closely related to, and probably derived from normal cellular genes - the protooncogenes. The dramatic transforming potential of protooncogenes can be attributed to the important roles that they play in growth and differentiation control pathways. In fact some have been identified as encoding growth factors, morphogens, and growth factor receptors.⁶⁸

Sequences associated with the β -actin gene contain several copies of an enhancer that is homologous to the serum response element(SRE) of the c-fos protooncogene. The actin and c-fos SRE's bind the same serum response factor(SRF) that

mediates transient transcriptional activation of both genes in response to serum mitogens.^{51,52,53} The DNA binding component of the SRF has been purified by its affinity for the SRE sequence, and identified as a 67 Kd nuclear protein.⁶⁹

Protein synthesis inhibitors in combination with mitogens result in a superinduction of the c-fos and β -actin genes, caused by an increase in the magnitude and duration of transcription.^{70,71} This suggests the existence of a labile transcription repressor.

The response of actin gene expression to mitogens is more complicated than that of the c-fos gene. β -actin mRNA is expressed at a higher constitutive level and becomes reinduced about one hour after the initial transient response.⁷² This might have something to do with the multiple SRE's of the β -actin gene, each with different degrees of homology to the c-fos SRE and which probably result in different protein binding properties.

The experiments of Leavitt, discussed previously, have indicated that expression of a mutant β -actin gene contributes to the neoplastic transformation of cells.³⁸ It is not clear whether this is caused by the mutant actin protein or by the altered expression of the normal β and γ cytoskeletal actins, which are coregulated.^{39,40} In either case it is clear that cytoskeletal actins are more than merely structural proteins in the cell, and that their expression, particularly that of β -actin, is intimately connected with changes in the cellular state of proliferation

and differentiation.

It is likely that changes in β -actin expression would take place in cells as they respond to proliferative and differentiative signals during urodele limb regeneration. A better understanding of the control of these processes would result from studies of cytoskeletal actin expression in the regenerating limb. Thus, the primary objective of this thesis was to isolate a β -actin homologous sequence from N. viridescens for use as a molecular probe in studies of cytoskeletal actin expression during limb regeneration.

MATERIALS AND METHODS:

General techniques for creating a genomic library and for cloning DNA sequences from it have been described in various comprehensive molecular techniques laboratory manuals. Three such books used extensively for this project were those of Maniatis et al⁷³ Davis et al,⁷⁴ and Berger and Kimmel.⁷⁵ Some procedures were modified from these sources, and a few were obtained from other sources. In many cases several different protocols were tried. The most successful protocols were adopted for further use and are included here. Only brief descriptions are given.

A simplified outline of the process of cloning specific DNA sequences from a genomic library is illustrated in Fig.6. The subcloning step is elaborated in Fig.7.

CHEMICALS, ENZYMES and BACTERIAL STRAINS

All chemicals and enzymes were commonly available from many suppliers. Often a single product was obtained from several suppliers. For these reasons the source of only a few materials will be included in the text. Bacterial strains used are listed in Table 3.

Fig.6

This figure diagrams the procedure for creating a genomic library and cloning a specific DNA sequence from the library.

The stuffer fragment of EMBL3 phage DNA is removed by restriction digestion. This is ligated to genomic DNA which has been partially digested with a restriction enzyme, to produce random fragments. The recombinant phage DNA is then packaged into bacteriophage coats producing the genomic library. Host bacteria infected with the phage are plated. Plaque lift membranes made from the plates are hybridized with a radioactive probe to identify plaques of phage containing the gene of interest. These plaques are replated several times to establish pure clones, which can then be restriction mapped and subcloned into plasmids.

S- Sall, B- BamH1, E- EcoR1

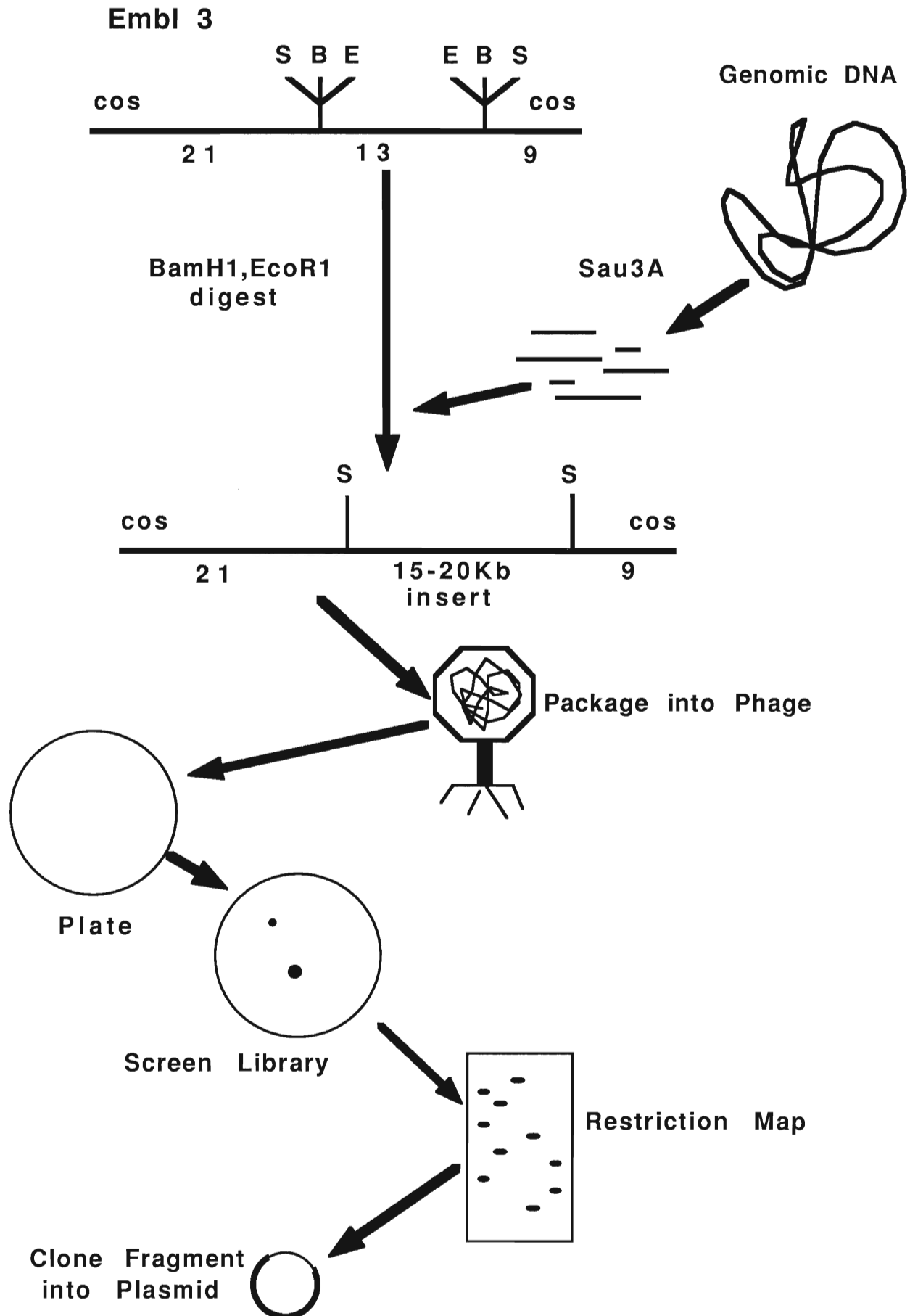


Fig.7

This figure diagrams the process of subcloning a specific DNA sequence from a lambda phage clone into a plasmid vector.

The phage DNA is restriction enzyme digested and fragments of different length are separated by agarose gel electrophoresis. The fragment of interest is cut out of the gel, purified, and ligated to plasmid DNA which has been digested with the same restriction enzymes, and dephosphorylated to prevent religation. The recombinant plasmid DNA is transformed into a suitable host bacterium. Bacteria containing plasmids are selectively grown by virtue of a plasmid antibiotic resistance gene. When using a vector with the *lacZ* gene, insert containing plasmids can be identified by white instead of blue colony colour. The *lacZ* gene encodes the enzyme, β -galactosidase, which breaks down colourless X-gal, included in the medium, to form a blue pigment. Inserts in the multicloning site disrupt *lacZ* resulting in white colonies. Insert containing clones are replated to produce pure clones. These can then be screened by restriction fragment analysis to confirm that they contain the desired insert.

Table 2

Escherichia coli strains which were used in this project as hosts for EMBL3 bacteriophage or pGEM1 and pTZ19R derived plasmids.

STRAIN	GENOTYPE	SOURCE	USE
HB101	F^- , <i>hsdS20</i> , <i>recA13</i> <i>ara14</i> , <i>proA2</i> , <i>lacY1</i> , <i>galK2</i> , <i>straA xyl-5</i> , <i>mtl-1</i> , <i>supE44</i>	gift of J. Rossant	highly transform- able strain, host for pGEM plasmids used as probes
Q539	<i>hsdR_k⁻</i> , <i>hsdM_k⁺</i> , <i>supE</i> , $\phi 80$, P2	gift of J. Rossant	host for EMBL3 bacteriophage
MB406	<i>supE</i> , <i>recB21</i> , <i>recC22</i> , <i>sbcB15</i> , <i>hflA</i> , <i>hflB</i> , <i>hsdR⁻</i>	gift from Promega	host for EMBL3 phage used to reduce loss of library clones
DH5 α	F^- , <i>endA1</i> , <i>hsdR17</i> , <i>supE44</i> , <i>thi-1</i> , λ^- , <i>recA1</i> , <i>gyrA96</i> , <i>relA1</i> , $\Delta(\arg F-lacZ\alpha)U169$, $\phi 80dlacZ$ M15	Bethesda Research Lab.	host for pTZ19R plasmid subclones
LE392	F^- , <i>hsdR514</i> (<i>r_k⁻</i> , <i>m_k⁺</i>), <i>supE</i> , <i>supF</i> , <i>lacY1</i> , <i>galK2</i> , <i>galT22</i> , <i>metB1</i> , <i>trpR55</i> , P2	not used	host for EMBL3, used by J. Brockes

SOLUTIONS

The following is a list of abbreviations for commonly used solutions. All other solutions are described fully in the references.

TE - 10mM Tris-HCL, 1mM EDTA, pH7.4

Phenol/chloroform - phenol: chloroform: isoamyl alcohol,
25:24:1

LB medium - 10g bacto-tryptone, 5g yeast extract, 10g sodium chloride in 1 litre water, pH7.2

10X SSC - 1.5M sodium chloride, 0.15 M sodium citrate, pH7.0

1X TBE - 0.89M Tris-HCL, 0.89M boric acid, 20mM EDTA, pH8.3

50X Denhardt's solution - 1% w/v ficoll(M.W.400,000),
polyvinyl-pyrrolidone(40,000), bovine serum albumin

1X SSPE - 180mM NaCl. 10mM sodium phosphate, 1mM EDTA, pH 8.0

THE GENOMIC LIBRARY AND MOLECULAR PROBES

An *N. viridescens* genomic library was prepared and kindly provided by J.Brockes(Ludwig Cancer Institute, London). DNA from a single newt was partially digested with the Sau3A restriction enzyme, then fractionated by NaCl gradient sedimentation to select 15-20Kb fragments. These were ligated into the EMBL3 lambda bacteriophage vector at BamH1 sites as shown in Fig.6, using the procedure of Frischauf.⁷⁶ The initial library size after packaging was 8×10^6 plaque forming units(pfu). This was amplified once, by plating with the bacterial host LE392(P2). We were provided with 0.5ml of lysate from this primary amplification. The titre was 1.7×10^9 pfu/ml.

Plasmids used as molecular probes are diagrammed in

Fig.8. These were gifts of J.Rossant (Mount Sinai Research Institute, Toronto). The β -actin⁴⁸ and oncogene sequences^{77,78} in the plasmids have been described.

PURIFICATION OF GENOMIC DNA

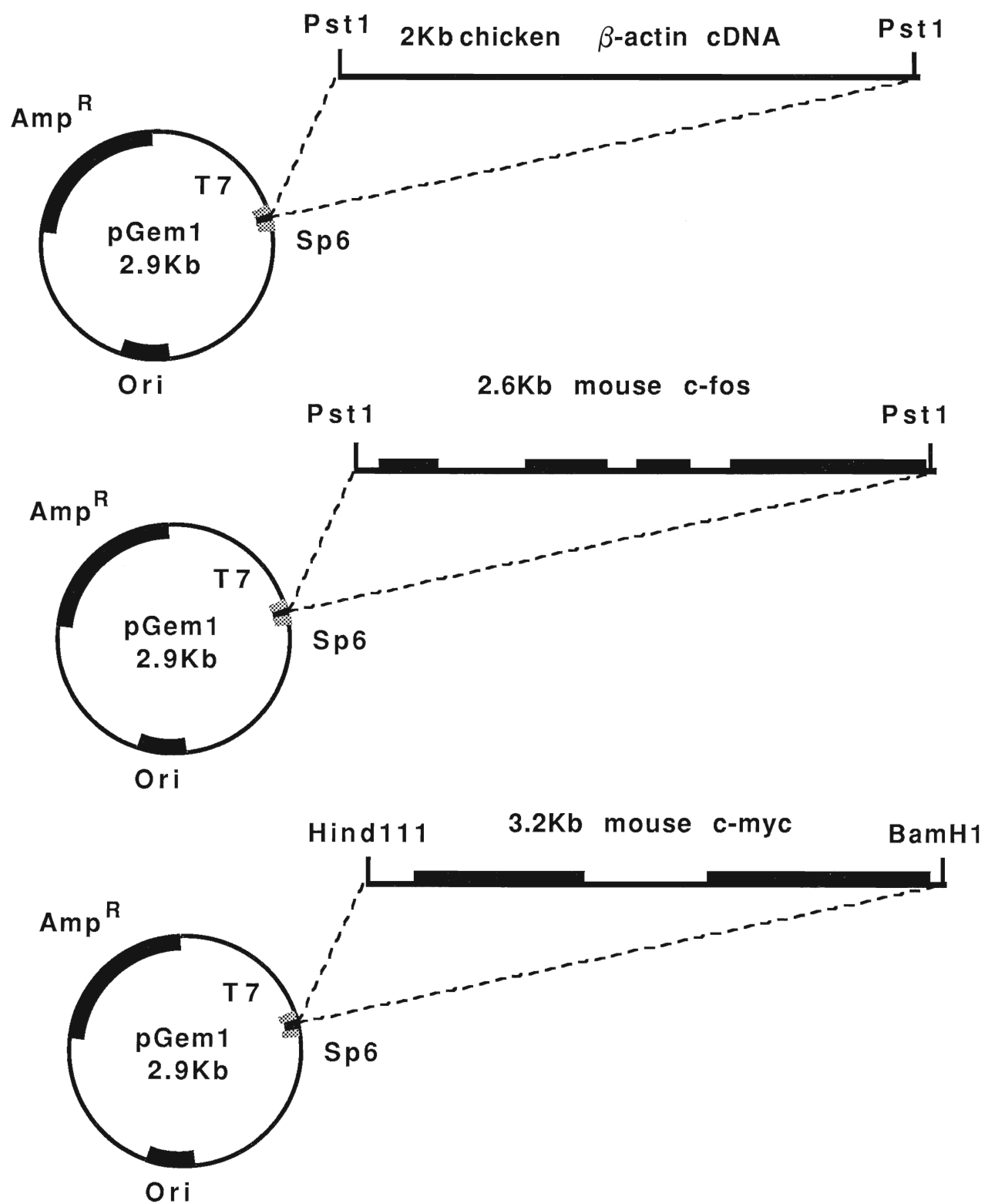
Genomic DNA was purified by the procedure of Maniatis.⁷³ Tissue was frozen in liquid nitrogen and ground to a fine powder with a precooled mortar and pestle, then disrupted in Proteinase K buffer, and digested overnight at 65°C with Proteinase K and DNase-free RNase A. The proteins were extracted with phenol/chloroform and the DNA was precipitated by addition of isopropanol then removed by spooling on a glass capillary tube. The DNA was dissolved in TE buffer, reextracted with phenol/chloroform, precipitated with ethanol and 5M sodium acetate, then dissolved in TE. Yeast (Saccharomyces cerevisiae) DNA was a gift from N. Ondrusek.

EMBL3 BACTERIOPHAGE DNA PURIFICATION

The most important factor in obtaining a good yield of EMBL phage DNA was found to be the titre of phage in the lysates. Using the method of Miller,⁷⁹ LB medium with 10mM MgSO₄ was prewarmed to 37°C and inoculated with the host bacterium(Q539 or MB406) to give an absorbance of less than 0.2. This culture was inoculated with 10⁹ phage/l when the absorbance reached 0.2. The culture was incubated at 37°C with vigorous shaking until it cleared due to cell lysis. If this did not occur, the culture could often be saved by a two-fold dilution with LB medium with 10mM MgSO₄.

Fig.8

The three plasmids used as molecular probes are diagrammed. The β -actin, c-fos and c-myc gene sequences are inserted into the multicloning site of the multipurpose plasmid pGEM1. This plasmid has ampicillin resistance and the RNA transcription promoters Sp6 and T7 flanking the multicloning site. Exons in the mouse sequences are indicated by a heavy line.



After lysis, chloroform was added and the incubation with shaking continued for 10 minutes to lyse remaining infected cells. The cell debris was removed by 10,000 rpm centrifugation. Two methods were used to purify DNA from liquid lysates: polyethylene glycol(PEG) phage precipitation or cetyl-trimethyl ammonium bromide(CTAB) DNA precipitation.

a. PEG Precipitation:

Using the method of Davis⁷⁴, phage were precipitated from the lysate by addition of PEG and NaCl. After digestion of E. coli DNA by DNase, the protein was removed by phenol/chloroform extraction in the presence of EDTA. DNA was then ethanol precipitated, dissolved in TE, reextracted and precipitated, then dissolved in TE. RNA was removed by digestion with DNase-free RNase.

b. CTAB Method

This procedure, modified from one recently described by Manfioletti and Schneider,⁸⁰ was quicker and easier than the standard PEG precipitation method which it replaced. E. coli DNA in the lysate was digested with DNaseI. The phage coat was digested with Proteinase K in the presence of EDTA and the EMBL phage DNA precipitated by addition of CTAB and centrifugation. The pellet was resuspended in 1.2M NaCl to displace the CTAB and then the DNA was ethanol precipitated. It was further purified by DNase free RNase digestion, phenol/chloroform extraction, and ethanol precipitation.

PLASMID DNA PURIFICATION

a. Large Scale

Large scale plasmid preparation was used to produce plasmid DNA for vectors and probes. The alkaline lysis method was performed as described by Maniatis.⁷³ This procedure makes use of lysozyme, SDS, and NaOH to lyse the bacterial cells. Protein and E. coli DNA were precipitated together by addition of a potassium acetate/formic acid solution and centrifugation. The plasmid DNA in the supernate was isopropanol precipitated, resuspended in TE and further purified in a CsCl₂ gradient with ethidium bromide(EtBr). This yielded up to 26mg of highly pure plasmid DNA per litre of chloramphenicol amplified culture.

b. Medium Scale

Medium scale plasmid isolation was done using the boiling method of Maniatis.⁷³ Bacterial cells were lysed by lysozyme, Triton X100 detergent, and limited boiling. The denatured proteins, together with the E. coli DNA are removed by centrifugation. Plasmid DNA in the supernate was digested with DNase-free RNase, extracted with phenol/chloroform, ethanol precipitated, and dissolved in TE. This yielded 50-100µg of plasmid DNA from a 50ml culture.

c. Small Scale

For analysis of the many bacterial clones produced by transformation, plasmid DNA was purified from 2ml cultures by a modified boiling procedure, using glass powder(obtained as a kit: Gene Clean glass milk from Promega). The protocol is outlined in the kit. It is similar to the medium scale

procedure above, except that the plasmid DNA was removed from the supernate by binding to the glass powder. Neither organic extraction or RNA digestion is necessary to produce DNA of sufficient quality for restriction enzyme digestion. The yield was about 4 μ g from a 2ml culture.

PURIFICATION OF E. COLI CARRIER DNA

E. coli B cells were grown in LB medium, incubated in a 37°C water bath. They were sedimented by centrifugation at 2000 g and resuspended in TE buffer, 1g/5ml. 0.5ml of 20% SDS was added, the solution was vortexed and put in a 60°C water bath for 10 min.. The solution was again vortexed, 1.4ml of 5M sodium perchlorate was added, and the solution was gently mixed. 7ml chloroform/isoamyl alcohol(24:1) was added to the mixture in a bottle and shaken for 10 min..(venting occasionally) After centrifugation at 10,000 g, the aqueous layer was removed to a clean tube and layered with 15ml ice cold 95% ethanol. The DNA was removed by mixing the solution with a glass rod, spooling the fibres onto the rod. The DNA was dissolved in TE.

DOT BLOT

The dot blot was performed by the procedure of Berger.⁷⁵ Alkali denatured DNA was spotted onto a nylon membrane using a vacuum blotting apparatus manufactured at Brock University.

AGAROSE GEL ELECTROPHORESIS

Electrophoresis was done using standard techniques described by Maniatis.⁷³ Agarose gels were prepared with 1X TBE buffer and in most cases, 0.8% agarose. Electrophoresis was carried out in 1X TBE buffer using horizontal gel boxes made at Brock University. Gels were then stained by soaking in EtBr solution (0.5 µg/ml) and photographed using a Polaroid camera and a UV transilluminator.

SOUTHERN BLOT

Although it specifically refers to the transfer of DNA from a size separation gel to a nylon or nitrocellulose membrane, the term Southern blotting or Southern analysis is usually used to describe the whole process of restriction enzyme digesting DNA, separating the DNA fragments by size, immobilizing the DNA on a membrane, and hybridization of the membrane with a radioactive probe. This procedure is diagrammed in appendix II.

DNA transfer was performed using the capillary blot protocol found in the Gene Screen Plus manual (New England Nuclear (NEN)). DNA in the agarose gel was denatured in 0.4M NaOH, neutralized, then transferred by the capillary action of 10X SSC buffer drawn up through the gel and membrane into paper towels. DNA carried out of the gel is trapped and bound to the membrane. This binding is permanent once the membrane dries. The membrane was either nylon or nitrocellulose. Nitrocellulose and some nylon membranes were baked for 1 to 2 hours at 80°C in vacuo to completely dry

them, according to the manufacturer's instructions.

PLAQUE LIFT

This procedure, described in Maniatis,⁷³ was used to transfer DNA from phage plaques on an agar plate to a nitrocellulose membrane. Phage and the host bacteria were spread on LB agar plates containing 10mM MgSO₄, and grown overnight at 37°C. A dry membrane was carefully placed on the chilled(4°C) plate. As it became wetted, unpackaged phage DNA from the plaques was drawn onto the membrane. The DNA was denatured, by soaking the membrane in alkali buffer, neutralized and fixed to the membrane after air drying, by baking in vacuo at 80°C. After hybridization and autoradiography, spots on the film were correlated with plaques, using asymmetric holes that had been punctured through the membrane into the plate with an india ink filled syringe.

RADIOACTIVE LABELLING OF DNA FOR PROBES

For the initial genomic and EMBL phage DNA Southern blots, and the library screening, the entire plasmid was used as a probe. It was labelled by nick translation using α -³²P-dCTP, according to the procedure of Maniatis.⁷³ Typically the specific activity of the probe was 10⁸ cpm/ μ g DNA.

For subsequent experiments, the 2Kb PstI fragment, containing the chick β -actin cDNA, was purified prior to labelling. (see RECOMBINANT DNA TECHNIQUES) This fragment

was radioactively labelled by the random primer technique using a kit from Bethesda Research Laboratory(BRL). Specific activity of this probe was greater than 10^9 cpm/ μ g DNA.

In both cases, labelled DNA was separated from unincorporated nucleotides by chromatography through Sephadex G50 in a spun column.(Maniatis⁷³)

HYBRIDIZATION

Molecular hybridization is the formation of double-stranded nucleic acid molecules by sequence-specific base pairing of complementary single strands. In this project, radioactively labelled DNA probes were denatured and hybridized to denatured DNA bound to membranes, in order to detect sequences complementary to the probe. The stringency of hybridization refers to the degree of sequence mismatching by the hybridization conditions: for example, under stringent conditions, the probe DNA will only hybridize to membrane-tethered DNA with a high degree of complementarity. Similarly post-hybridization washes may be done with different degrees of stringency. The kinetics of DNA hybridization and factors that affect hybrid stability are discussed in appendix III. The major factors that determine stringency are temperature, sodium concentration, and formamide concentration of the hybridization and wash solutions.

In this study, there was expected to be substantial sequence divergence between the probes and target DNA because they were from different species. For this reason,

hybridizations and washes were done at low stringency. In all cases, the hybridization solutions contained 0.1M sodium and 50% formamide. The washes contained 0.33M sodium with no formamide. The hybridization temperature was 37°C, and the wash temperature was from 37 to 50°C.

Two different hybridization solutions were used, differing mainly by the agents used to block non-specific binding of the probe to membranes. At the beginning of this study, nylon membranes were used for Southern blots and the dot blot. The hybridization solution contained 50% formamide, 1% SDS, 1M NaCl and 10% dextran sulfate as described in the Gene Screen manual.(NEN) Nitrocellulose was used for the primary library screening and all other Southern blots. Hybridization solutions contained Denhardt's solution, 50% formamide, 5X SSPE and 0.1% SDS, as described by Maniatis.⁷³

Two mg/10ml E. coli DNA was used as a nonspecific blocking agent in all hybridization solutions, instead of salmon sperm DNA which is most commonly used. This was done to prevent hybridization of the chick actin probe to salmon DNA: the salmon actin gene would not be much more divergent from the chick gene than the newt actin gene.

All hybridizations were carried out in heat sealed polypropylene bags or polypropylene sandwich boxes, submerged in a shaking water bath. Hybridizations which contained dextran sulfate were incubated overnight. Those without it were incubated for 48 hrs, as explained in appendix III.

PROBE REMOVAL

The radioactive probe was removed from hybridized membranes by soaking them in several changes of distilled water at 95-100°C.

AUTORADIOGRAPHY

Wet, hybridized blots were sealed in polypropylene bags and exposed to Kodak AR-X film at -70°C, with Cronex Lightning Plus enhancer screens. Either Sigma cardboard cassettes or Wolf cassettes were used.

The film was developed for 4 minutes with Kodak GBX developer, rinsed briefly in water, fixed for 2 minutes with Kodak GBX fixer, washed for 5-10 minutes in running water, then hung up to air dry.

RECOMBINANT DNA TECHNIQUES

Restriction enzyme digestion was carried out using standard buffers and temperatures recommended by Maniatis⁷³ or using buffers supplied with the enzymes upon purchase.

To obtain a particular DNA fragment, DNA was digested with the appropriate restriction enzymes and the fragments were separated by electrophoresis. The band with the required fragment was then excised from the gel and the DNA purified from the agarose. For less than 2µg of DNA, restriction fragments were purified by dissolving the agarose in NaI solution and binding the DNA to glass powder according to instructions that were included with the Gene Clean glass milk kit.(Promega) For larger pieces of gel or greater

quantities of DNA, the fragments were purified by electroelution into dialysis tubing . Electroelution, done as described by Maniatis,⁷³ was followed by phenol/chloroform extraction and ethanol precipitation of the DNA.

DNA fragments were ligated to plasmids using the protocol and solutions described by King and Blakesly.⁸¹ Plasmid DNA was dephosphorylated at the 5' ends using calf intestinal phosphatase, by the method of Maniatis.⁷³

CHOICE OF PLASMID VECTOR

The multipurpose plasmid pTZ19R(Pharmacia), shown in Fig.9 was chosen, for subcloning of newt fragments from EMBL phage, for the following reasons:

- 1) The small (2.9Kb) size allows efficient cloning of fairly large inserts.

- 2) There is an ampicillin resistance gene for positive selection of E. coli transformants containing the plasmid.

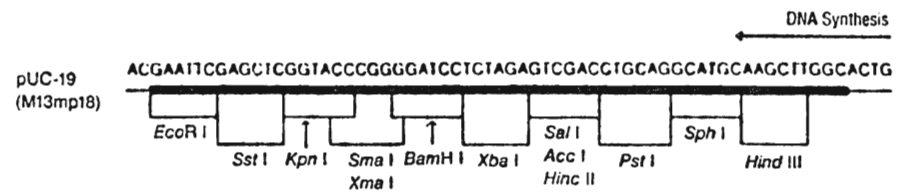
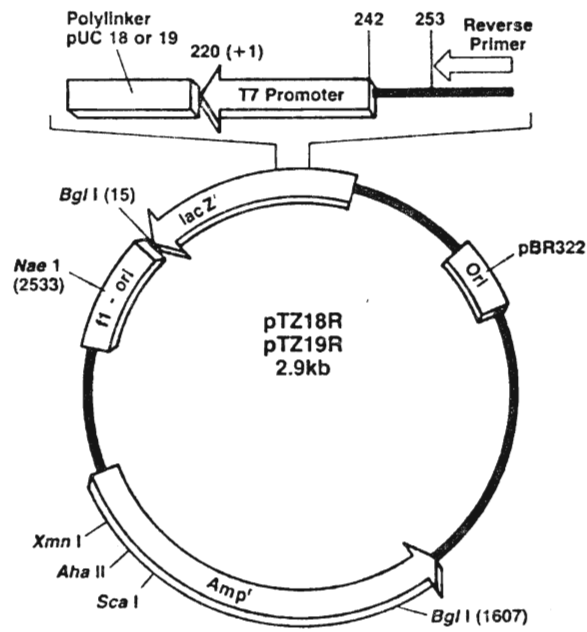
- 3) The puc19 multicloning site allows flexibility of restriction enzymes used to remove the chosen fragment from EMBL DNA. It also places convenient restriction sites next to the insert. These sites appear only once, simplifying restriction mapping.

- 4) The multicloning site interrupts a lac Z gene so that plasmids with inserts may be selected by their white colour on X-gal indicator plates, as opposed to the blue colour of non-insert bearing plasmids.

- 5) T7 and T3 RNA transcription promoters on either side of the multicloning site make it possible to produce RNA from

Fig.9

The multipurpose plasmid pTZ19R(Pharmacia), used in this project to subclone newt genomic DNA fragments. The orientation and sequence of the puc19 multicloning site is indicated as well.



either strand of the insert. Synthetic oligonucleotide primers, complimentary to each promotor sequence, are available to facilitate sequencing of the inserts from both directions as well as other applications.

6) Because pTZ19R carries a bacteriophage F1 origin of replication, in the presence of a 'helper phage' MK301, it can replicate as a bacteriophage (eg like M13), excreting single stranded DNA into the medium for use in sequencing and other applications.

BACTERIAL TRANSFORMATION

Frozen competent cells with a tranformation frequency of greater than 10^3 transformants/ng were inexpensive and readily available commercially. Competent DH5 α cells were purchased from BRL. This strain is suitable as a host for plasmids using the lac Z selection system and has the advantage of lacking recombination and restriction enzymes which can destroy plasmid inserts. DH5 α cells were transformed using the BRL protocol included with them. Two μ l of ligation mix was added to a 50 μ l aliquot of frozen cells, thawing on ice. The solution was gently mixed and kept on ice for 30 min.. The cells were then heat-shocked for 40 sec. at 42°C and returned to the ice for several minutes. 950 μ l of LB medium was mixed with the cells which were then incubated for 50 min. in a shaking 37°C water bath. The transformed cells were concentrated by spinning for 5 sec. in a microfuge, then spread onto LB plates containing ampicillin and X-gal. Control plasmid DNA(puc19) gave a

transformation frequency of 2×10^3 colonies/ng DNA.

Fig.10

Autoradiographs of a dot blot containing genomic DNA as labelled. The probe was removed between hybridizations.

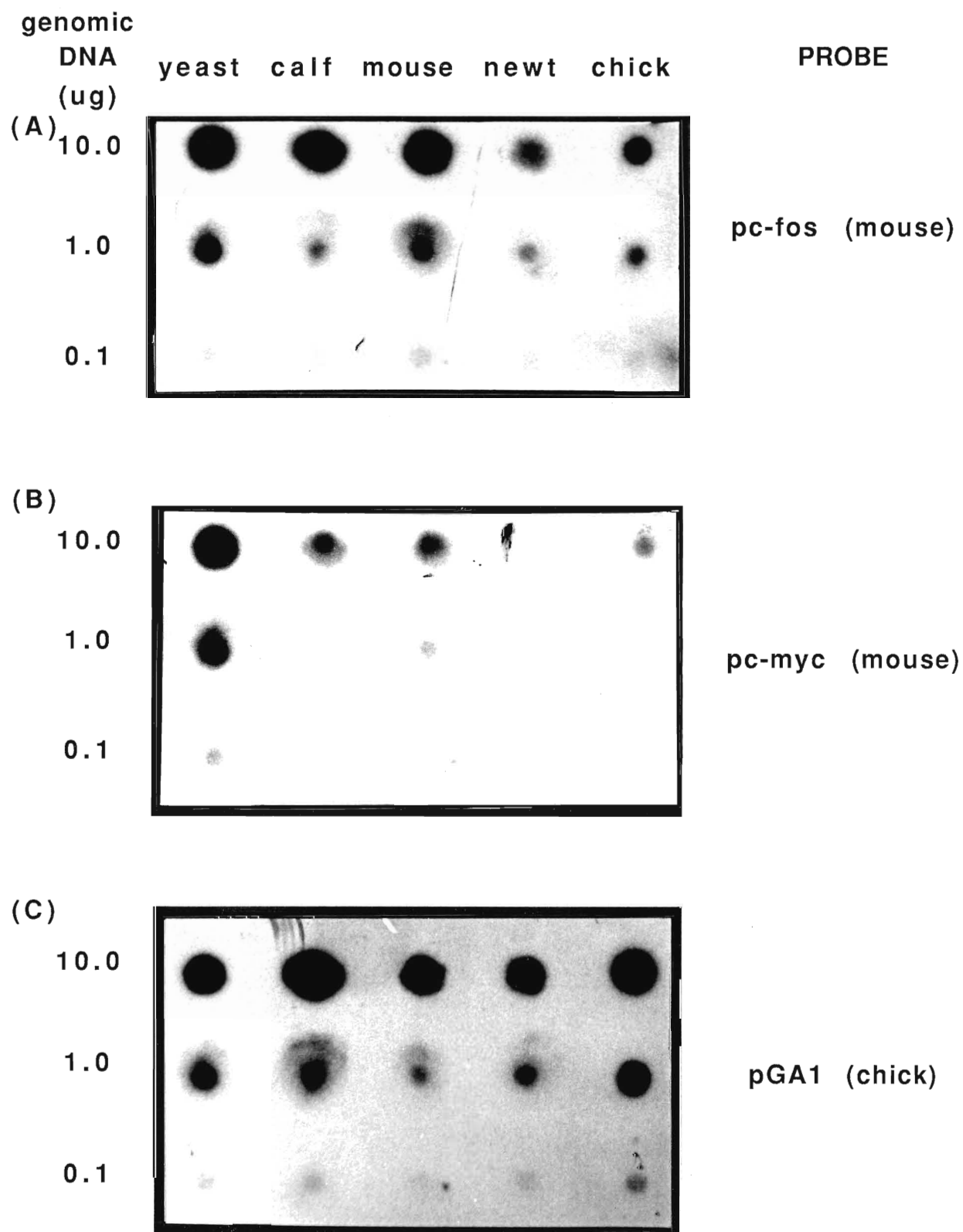
RESULTS:

Dot Blot

A newt genomic library was to be screened for c-myc, c-fos, and cytoskeletal actin genes using the plasmids shown in Fig.8 as probes. Since these probes were from different species than newt, (c-myc and c-fos were mouse genes, and the actin was from chicken) it was possible that they might not be homologous enough with newt genes to cross hybridize well.

In order to test the extent of hybridization of the probe plasmids to newt DNA, a dot blot was prepared containing 10ug, 1.0ug, and 0.1ug of DNA from yeast, calf, mouse, newt and chick. This blot was hybridized with each of the three radioactively labelled probes, as shown in Fig.10. The yeast strain carried a plasmid with some sequences identical to the probe plasmids, therefore serving as a positive control. Low stringency conditions were used for these and all other hybridizations except where noted.

The yeast DNA cross reacted well with each probe as expected, since it contained a plasmid with areas of homology to probe plasmids. The c-myc(Fig.10b) and c-fos(Fig.10a) probes showed significant cross reaction with the mammalian DNA, but hybridized less strongly to the chick DNA. The c-fos probe, but not c-myc, cross-reacted with newt DNA. The actin(Fig.10c) probe hybridized well with the DNA from all species.



SOUTHERN BLOTS

A Southern blot(Fig.11a,b) was also prepared using yeast, calf, mouse and newt DNA digested with EcoR1 and hybridized with the fos and actin probes, which had hybridized with newt DNA on the dot blot.~~three probes~~. This is a more rigorous test of homology with the probes because the banding pattern produced by genuine, sequence specific, hybridization can be distinguished from nonspecific binding of the probe. The c-fos probe hybridized to bands in the calf and mouse lanes. It didn't produce bands in the newt lane, as might have been expected from the dot blot. The actin probe hybridized to several bands in the calf and mouse lanes and possibly two or more in the newt lane. It also hybridized to a single band, in addition to the plasmid band, in the yeast lane.

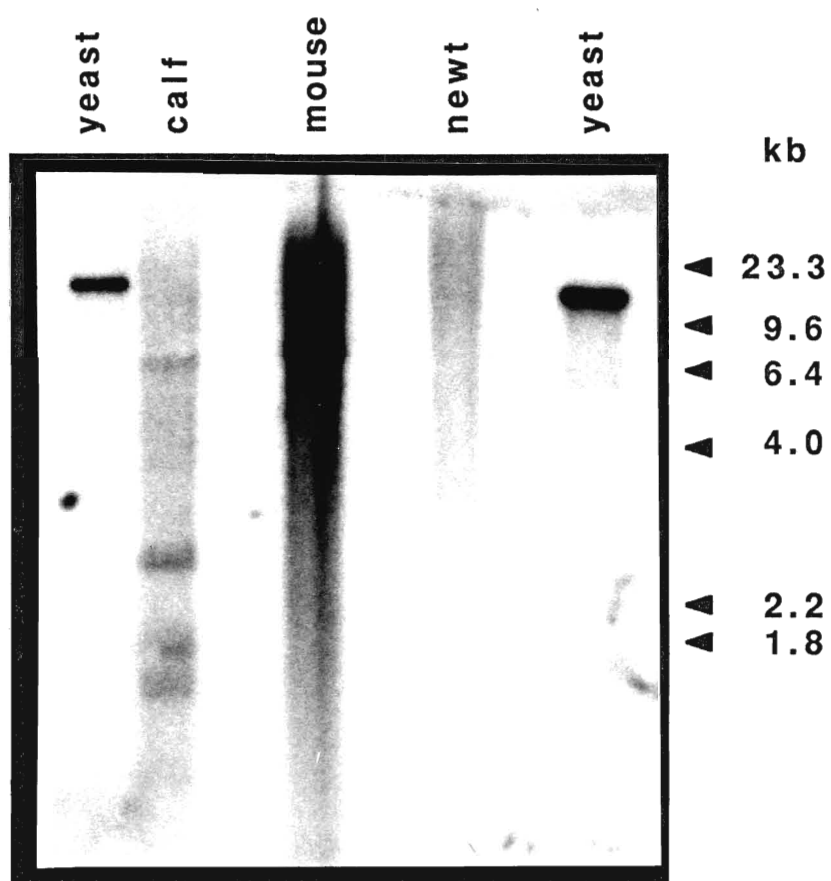
Two other Southern blots, prepared and kindly provided by Gordon Fraser,(in our laboratory) were also hybridized with the actin probe. The first blot contained DNA from seven different species, digested with Cfo1.(Fig.12) Bands hybridizing with actin were apparent for all species but newt. The second blot contained newt DNA digested with a variety of restriction enzymes. As was the case with newt DNA lanes in previous Southern, the probe hybridized to smears and discrete bands were not obvious.(data not shown)

Despite the difficulties with the genomic DNA Southern blots, a Southern blot was performed using DNA purified from the newt genomic library and digested with EcoR1 and Sall. Sall cuts the EMBL3 bacteriophage DNA at sites on either end

Fig.11a,b

Autoradiographs of the same Southern blot hybridized with both the fos(Fig.11a) and actin(Fig.11b) probes. The probe was removed between hybridizations. Each lane contained 20µg of genomic DNA from different species, as labelled. The DNA was digested with EcoR1.

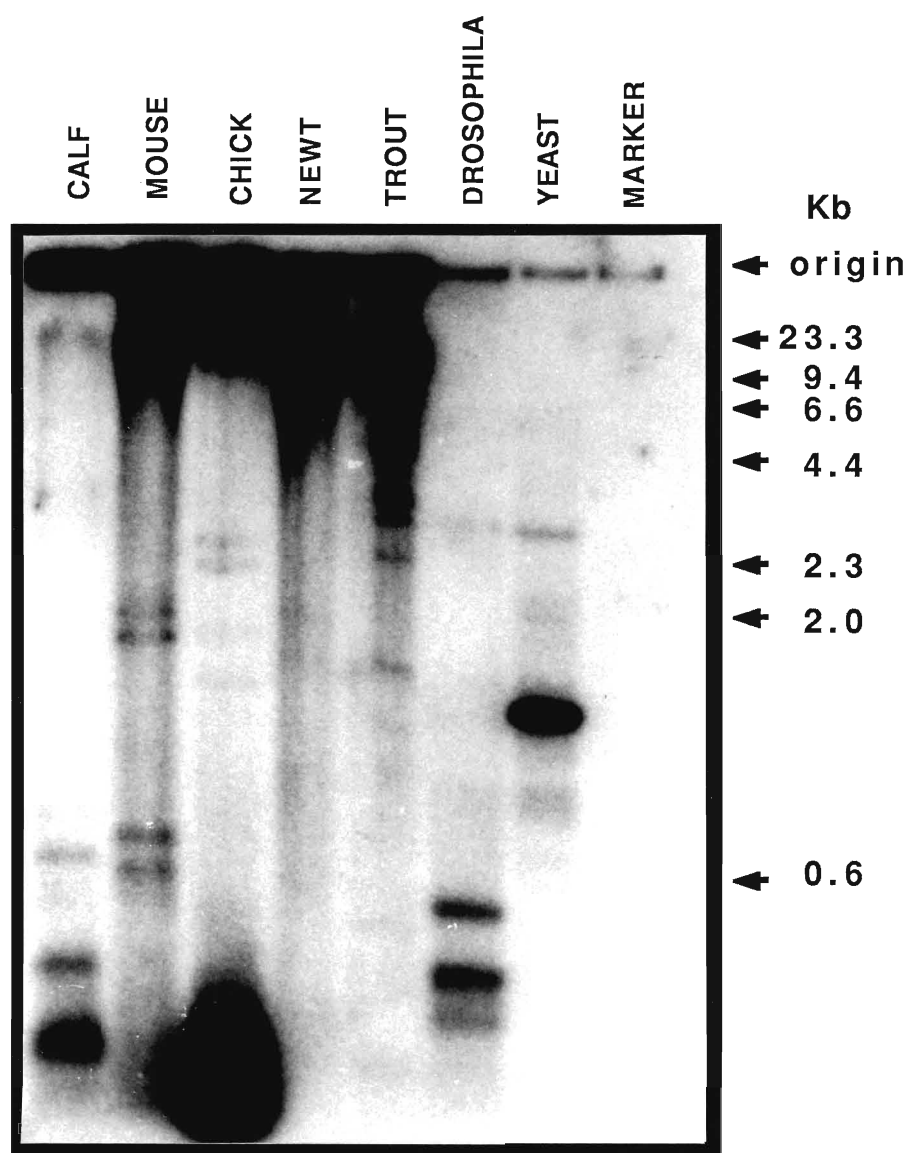
(A)



(B)

Fig.12

An autoradiograph of a Southern blot hybridized with the actin probe. Ten μ g of genomic DNA from different species was loaded per lane, as labelled. The DNA was digested with CfoI. This blot was prepared and kindly provided by Gordon Fraser.



Sall cuts the EMBL3 bacteriophage DNA at sites on either end of the inserted newt DNA, releasing the EMBL3 arms, evident in the EtBr stained gel.(Fig.13b) The inserts, cut at random EcoRI and Sall sites, form a smear of fragments. When hybridized with the actin probe, seven bands were clearly visible.(Fig.13a) This Southern blot was reprobed with the c-fos probe. The autoradiograph(Fig.14) revealed residual bands from the actin hybridization, but also one band not detected with the actin probe.

LIBRARY SCREENING

Because the actin probe hybridized well to several fragments of DNA from the genomic library, we decided to screen the library for actin. The very large amount of DNA in the newt genome required that 6×10^6 plaques should be screened to give a 95% chance of recovering a single copy gene.(see calculation, appendix I) As a start, the library was plated at a density of approximately 12,000 plaques/plate onto 50 plates, totalling approximately 6×10^5 plaques. The DNA from the phage plaques was transferred to nitrocellulose by the 'plaque lift' procedure. Hybridization with the actin probe identified an average of 2 positive plaques per plate,(Fig.15) twenty of which were selected for further screening. These impure plaques were replated at a density of 500 plaques per plate and rescreened. No positive signal from the autoradiographs which coincided with plaques could be detected, however. This was unexpected because a large proportion should have been progeny of the plaques which produced a positive signal in the first screening.

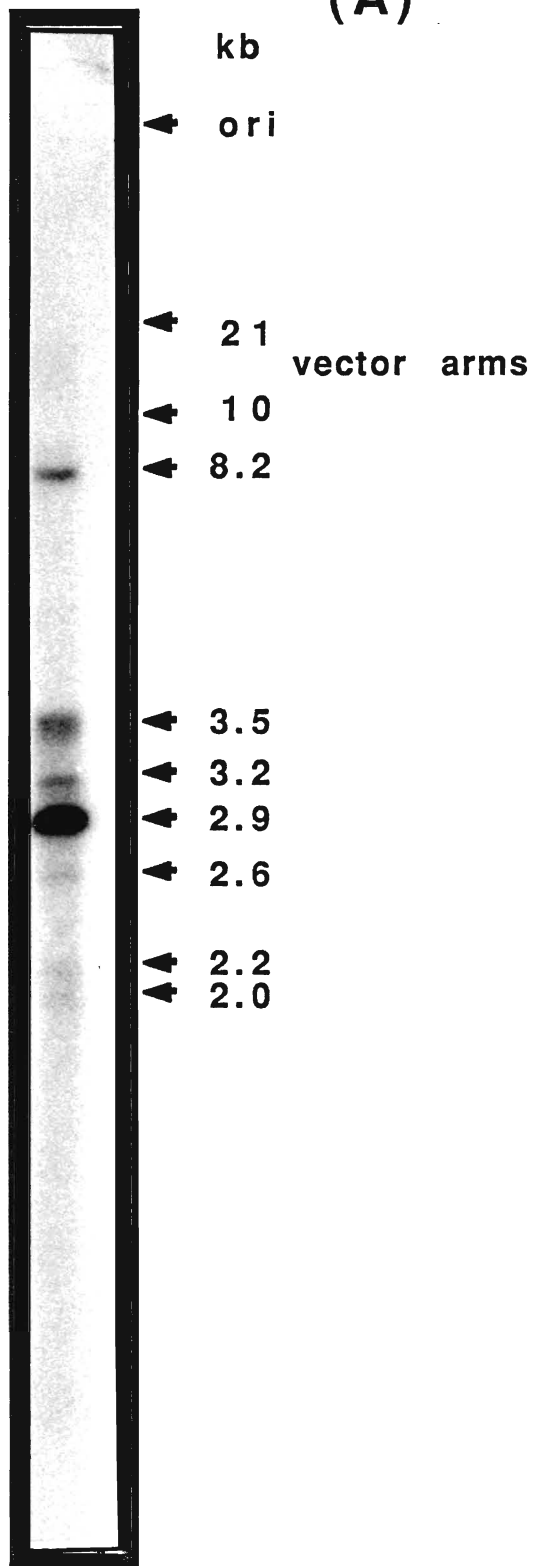
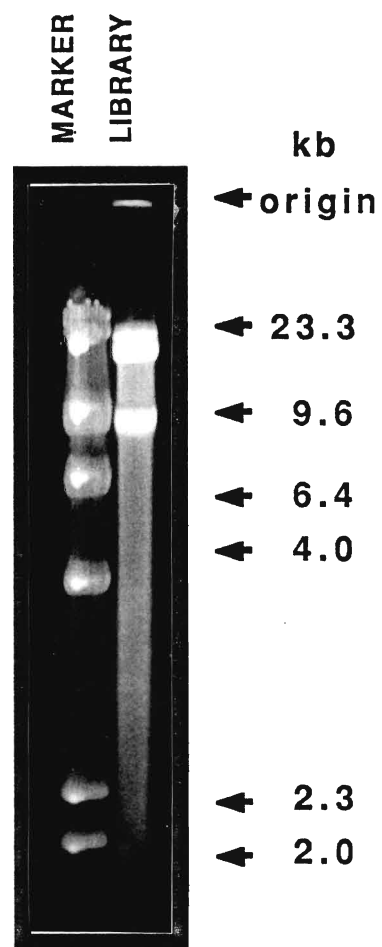
(A)**(B)**

Fig.14

An autoradiograph of the same Southern blot as in Fig.13a, but probed with the c-fos probe.

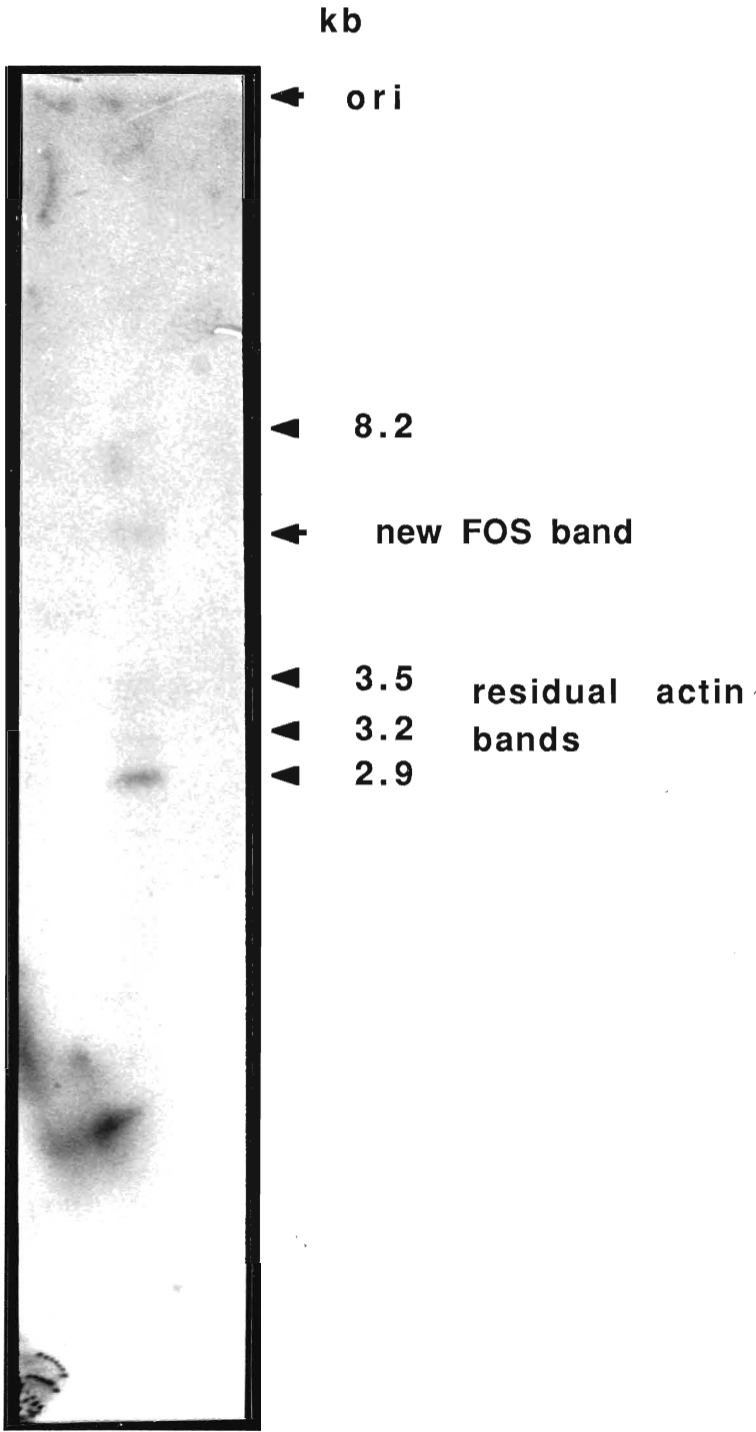
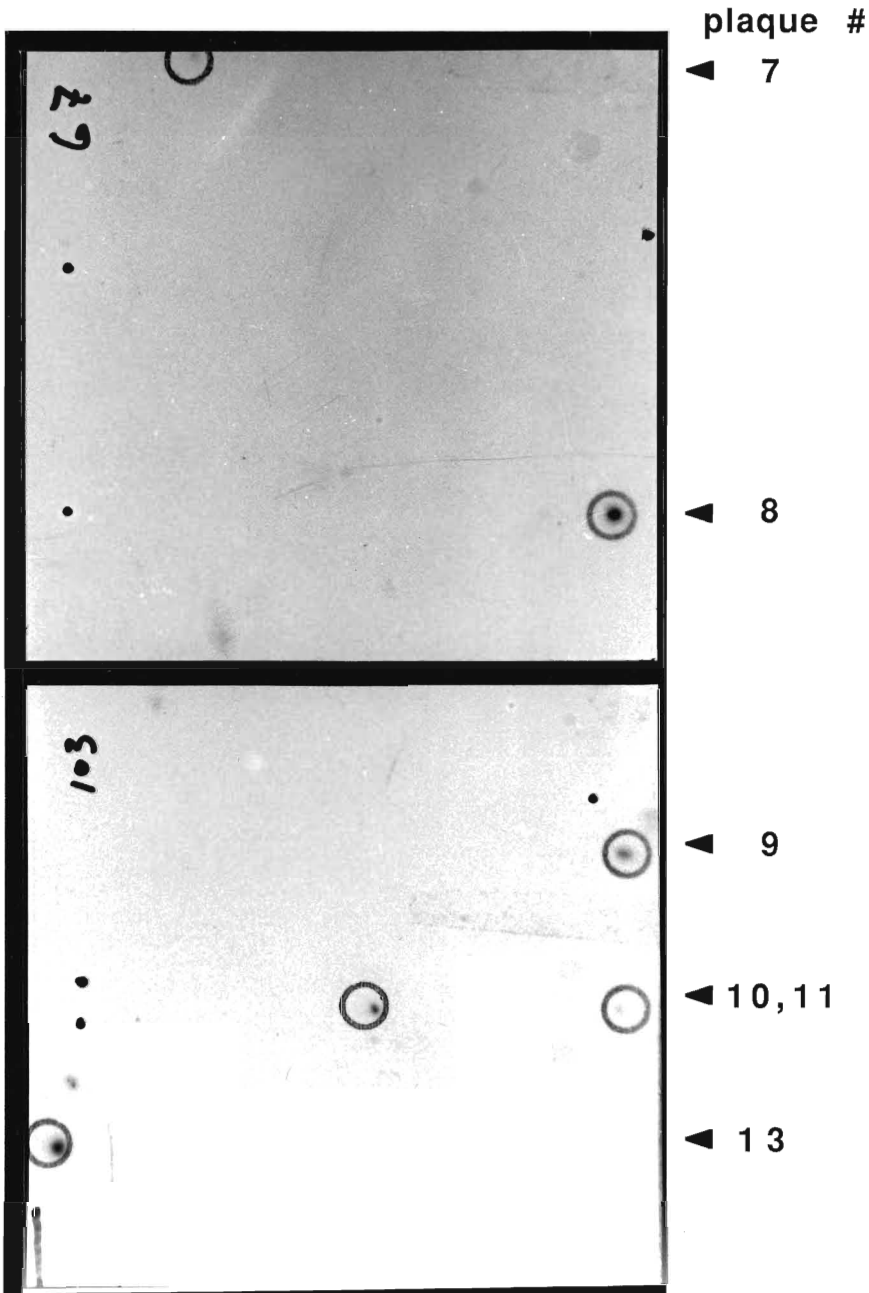


Fig.15

Two examples of primary plaque lifts probed with the actin probe. Some of the 20 positive plaques that were chosen for further examination are circled and numbered. The black marker spots indicate alignment holes that keyed the plaque lifts to the agar plates.



DNA was prepared from the 20 primary positive plaques, digested with EcoR1, and Sal1, Southern blotted and hybridized with the actin probe. The probe did not hybridize to bands from any of the DNA samples.(data not shown) Interpretation of the above two results was complicated by the presence of a high level of nonspecific background hybridization.

In an attempt to solve these problems, changes were made in the hybridization procedures. The 2 kilobase(Kb) actin cDNA fragment was purified from the plasmid for use as a more specific probe. Nitrocellulose was used for the blotting membrane rather than nylon. In the hybridization solution, Denhardt's solution was included as a nonspecific blocking agent and dextran sulphate was left out. The random primer technique was used instead of nick translation to label the probe to a higher specific activity and give more uniform probe quality.

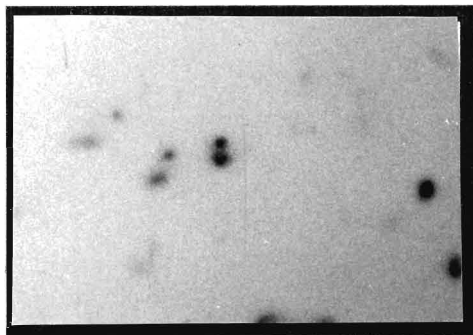
In addition to these procedural changes, a different host bacterial strain was used for phage propagation. This host was deficient in the recombination enzymes, RecBC and exonuclease I, thereby reducing the instability of the library during amplification.

Screening of the library for the newt actin gene was continued by replating several of the original 20 positive plaques. This time plaque lifts revealed that 20 to 30 percent of the plaques hybridized with the actin probe.(Fig.16a) DNA from 10 of these plaques was digested with EcoR1 and Sal1,(Fig.16c) and Southern blotted. Most of

Fig.16a,b,c

Fig.16a is an example of a plaque lift from the secondary screening of one of the primary plaques. Fig.16b shows the autoradiograph of a Southern blot prepared with phage DNA purified from plaques that were positive in the secondary screening. Fig.16c is the EtBr stained gel used to produce the blot. The numbers above the lanes correspond with isolated plaques. The amount of DNA varied between lanes.

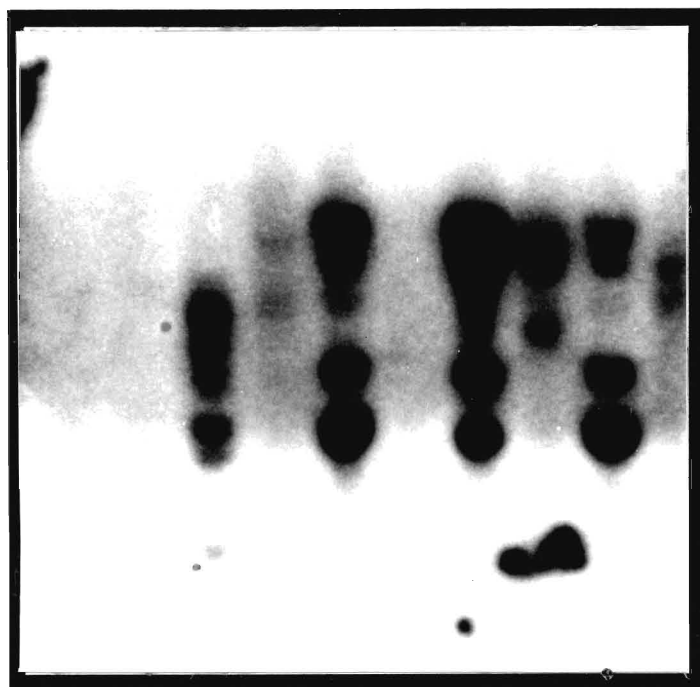
(A)



(B)

Plaque #

1 3 4 6 8 9 11 12 14 17



Kb

← 23.3

← 9.4

← 6.6

← 4.4

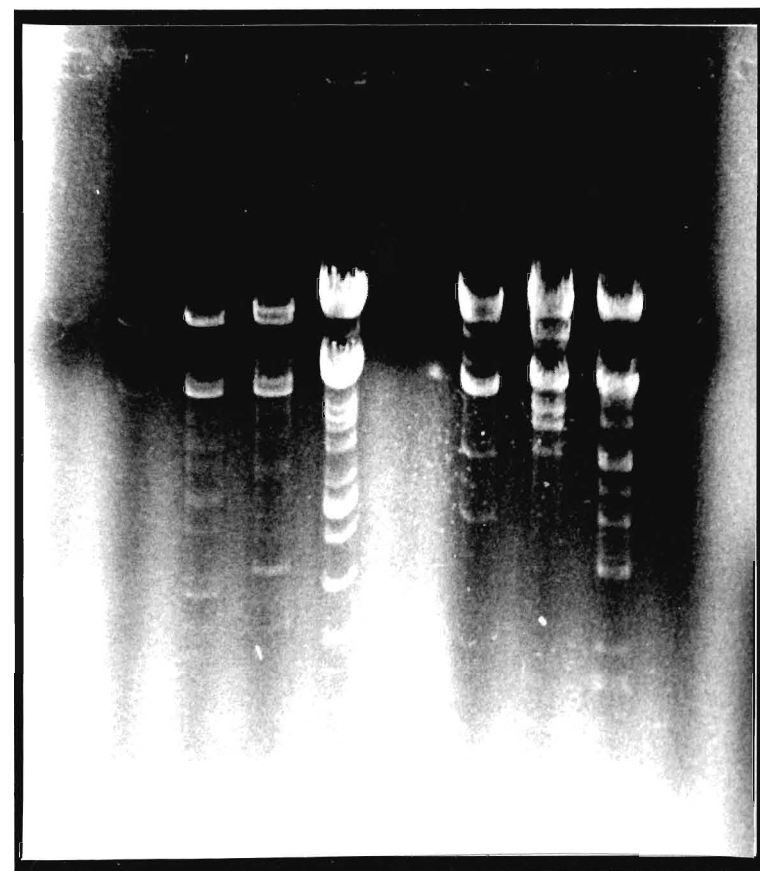
← 2.3

← 2.0

(C)

Plaque #

1 3 4 6 8 9 11 12 14 17



Kb

← 23.3

← 9.4

← 6.6

← 4.4

← 2.3

← 2.0

these DNA samples showed patterns of bands hybridizing to the actin probe.(Fig.16b) One of these plaques, well separated from neighbouring plaques, was replated at low density, plaque lifted and hybridized with the actin probe. All progeny of this plaque, #11, gave rise to actin-positive plaques. One of these 'plaque purified' clones, designated 11A, was selected for further analysis.

Concurrently with rescreening the twenty primary plaques, 20,000 plaque forming units(pfu) from the original library were plated and screened. A large number of strongly positive plaques were identified by the actin probe.(Fig.20a) One, designated 16, was put through two more screenings, resulting in the plaque purified clone 16AA.

RESTRICTION SITE MAPPING OF EMBL3 CLONES

The selected clones 11A and 16AA each consisted of a 15-17 Kb newt genomic DNA insert flanked by the 21 and 9.1 Kb EMBL3 phage arms. To identify the region of the insert containing actin sequence homology, restriction site mapping was performed. The restriction maps were also necessary for further subcloning of the actin homologous regions. Mapping utilized only those restriction enzymes which were known to have no recognition sites in the phage vector arms, (or one, in the case of HindIII) so that meaningful information could be obtained about the insert.

DNA from EMBL3 clone 11A was purified and digested with four different restriction enzymes, singly and in combinations. The DNA was then electrophoresed through agarose and stained with EtBr.(Fig.17a,18a,b) The gels were

Fig.17a,b

The plaques 11 and 17, analyzed in Fig.16b were rescreened to give pure phage clones 11A and 17A. This figure shows the EtBr stained gel(a) and autoradiograph(b) of the Southern blot prepared with DNA purified from phage clones 11A and 17A and hybridized with the actin probe. Two μ g of DNA was loaded per lane, after digestion with restriction enzymes as labelled above each lane. Some of the bands result from incomplete(partial) restriction enzyme digestion.(5.7Kb band in the Sall,BamH1 lane)

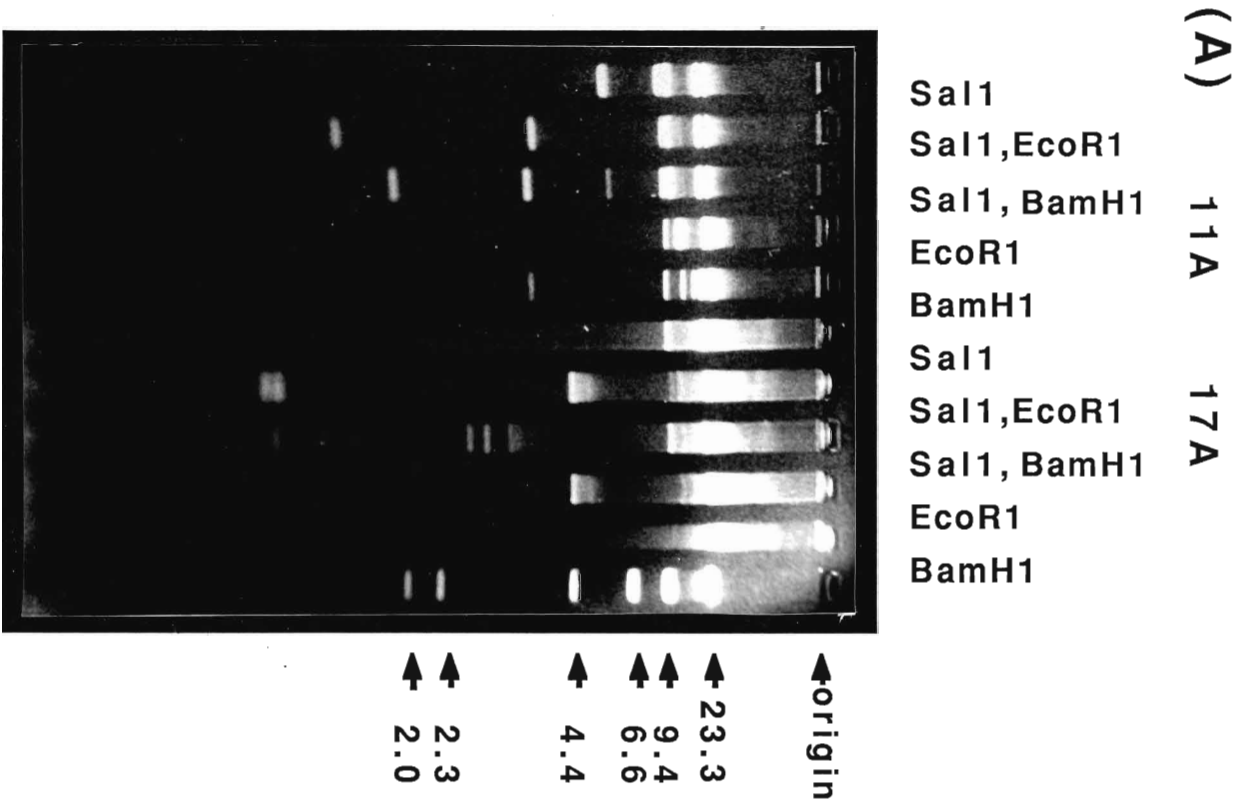
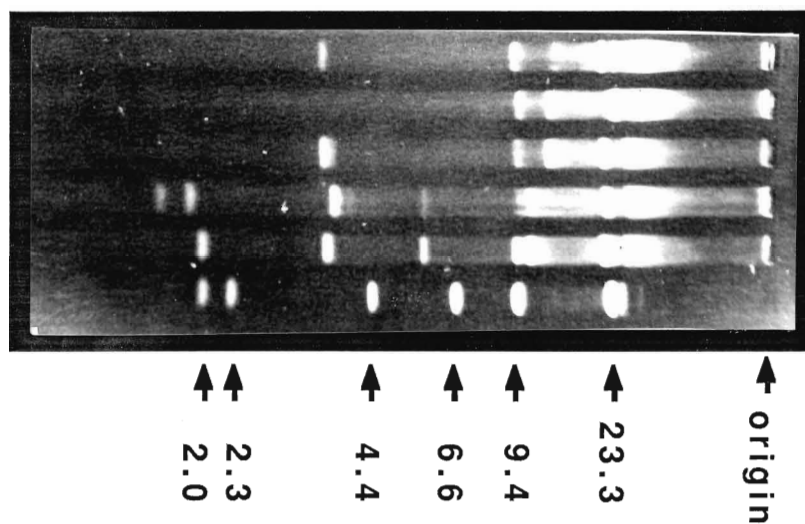


Fig.18a,b,c

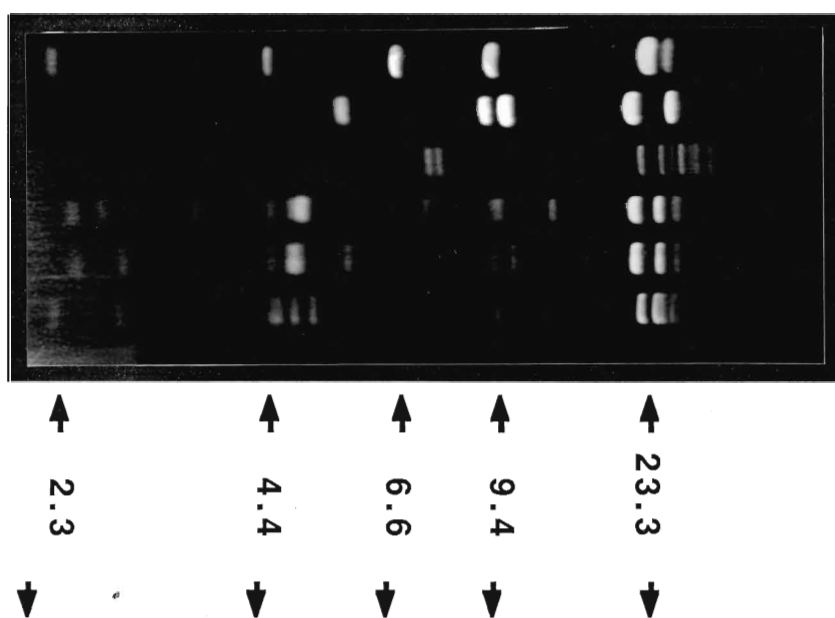
Fig18a,b show EtBr stained gels of DNA purified from phage 11A. Two μ g of DNA, digested with the indicated restriction enzymes, was loaded per lane. The autoradiograph of the Southern blot prepared from the gel in Fig.18b and hybridized with the actin probe is shown in Fig.18c.

(A)



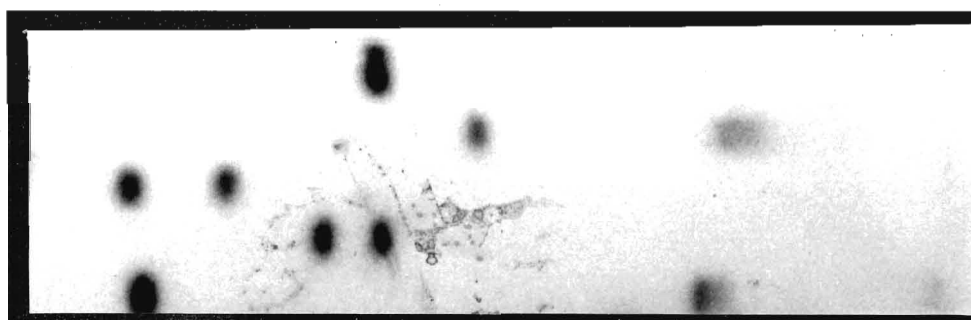
BamH1
EcoR1
BamH1,EcoR1
Sal1,EcoR1
Sal1,BamH1

(B)



Sal1
Hind111
Hind111, BamH1
Hind111,Sal1
Hind111,EcoR1

(C)



then Southern blotted and hybridized with the actin probe. From the size of the various bands in the EtBr stained gels and the identification on Southern blots(Fig.17b,18c) of those which hybridized to actin, it was possible to deduce the restriction map shown in Fig.19. The restriction and Southern analyses, shown in Fig.17,18, are just a few examples. These procedures were repeated using different restriction digestion and electrophoresis conditions to obtain reliable information. In some cases partial restriction digestions revealed sites not apparent from complete digestions. The sizes given in the restriction maps are estimates determined from several analyses by comparison to standard size markers. The margin of error for most fragments is less than 10%, as judged by the reproducibility of the size estimates.

DNA from the EMBL3 clone 16AA was mapped in the same way as 11A. An EtBr stained gel is shown in Fig.20c, and the Southern blot analysis of the same gel is shown in Fig.20d. The restriction map determined for EMBL3 clone 16AA is diagrammed in Fig.21. It is quite different from that of 11A, indicating that the two clones are distinct genomic DNA fragments. Another difference was that the autoradiograph shown in Fig.20d was exposed for only 3 hours, compared to that of 11A, Fig.17b,18c, which were exposed about 20 hours. This indicated that the 16AA newt sequence may be more closely related to the chick β -actin probe than the 11A newt sequence.

Fig.19

A restriction map of EMBL3 phage clone 11A, containing a 16 to 17Kb newt DNA insert with homology to the chick β -actin gene. The approximate region of homology is indicated. Thick lines represent the vector arms. Note the HindIII site in the right arm of EMBL3.

EMBL3 Clone 11A

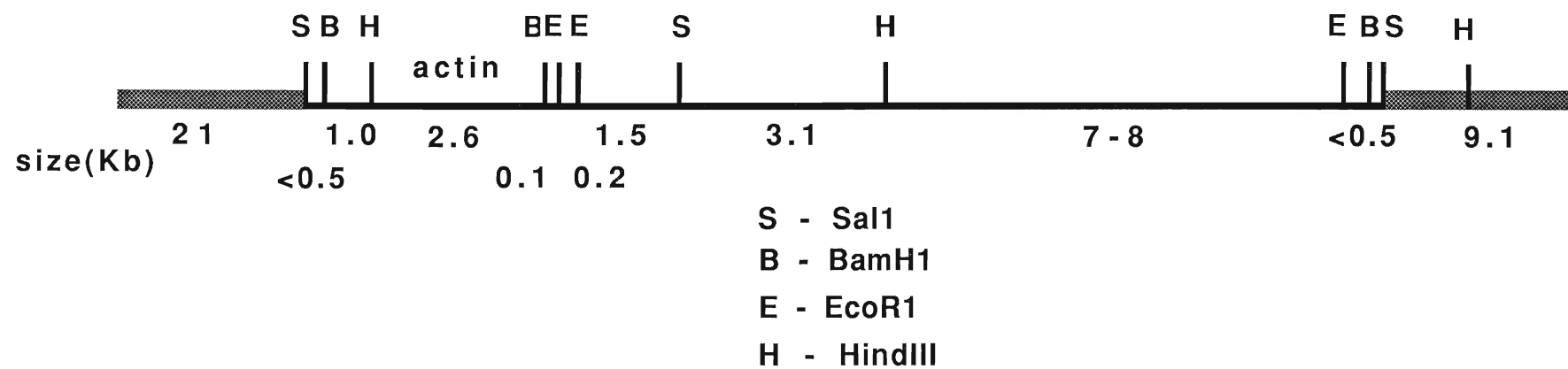
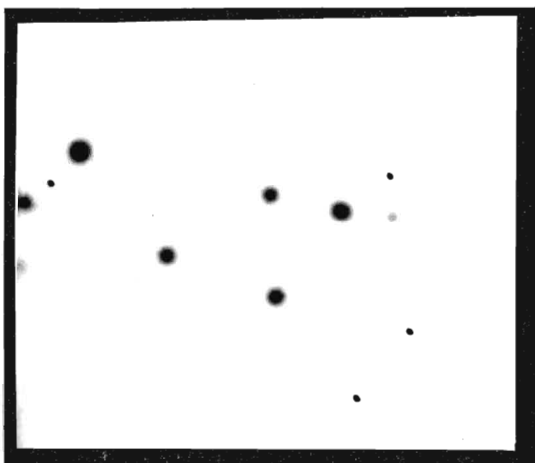


Fig.20a,b,c,d

Representative stages during the rescreening of the library. Fig.20a shows an autoradiograph of the primary plaque lift of the single plate which was inoculated with 20,000 pfu from the genomic library. The plaque with the strongest signal was designated #16 and further screened. Fig.20b is an autoradiograph of the tertiary plaque lift. All of the plaques were positive. One of these was designated 16AA. Fig.20c,d are the EtBr stained gel and autoradiograph of the Southern blot produced with DNA purified from the recombinant phage 16AA, which was selected from the tertiary screening. Two μ g DNA was digested with restriction enzymes as labelled above the lanes.

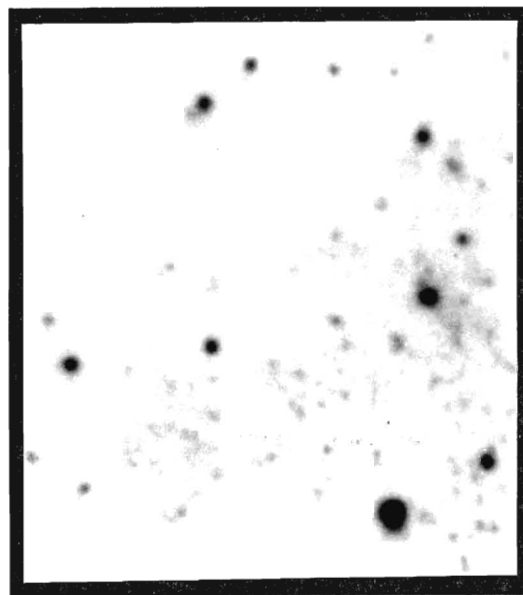
(B)

Tertiary Lift



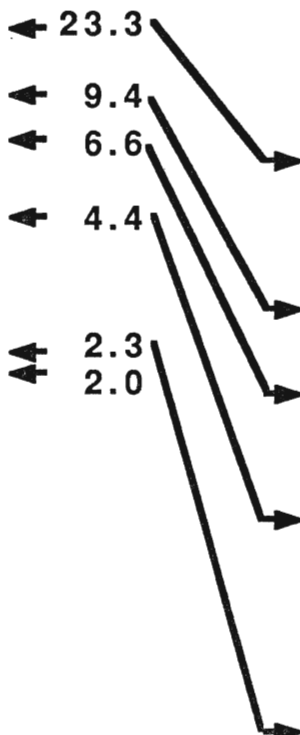
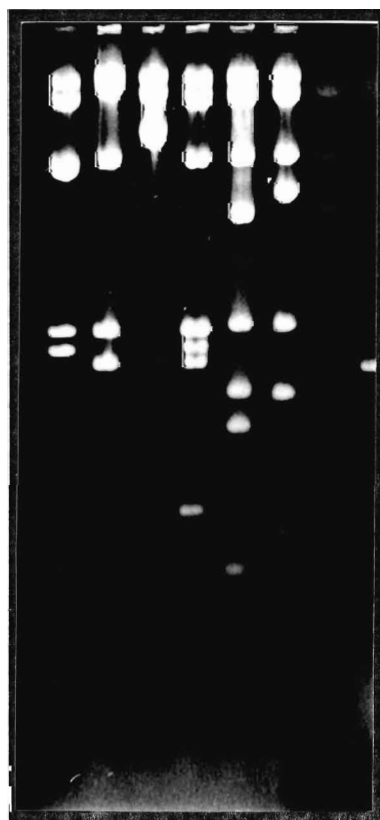
(A)

Primary Lift



(C)

SalI
EcoRI
BamHI
SalI,EcoRI
SalI,BamHI
BamHI,EcoRI



(D)

SalI
EcoRI
BamHI
SalI,EcoRI
SalI,BamHI
BamHI,EcoRI

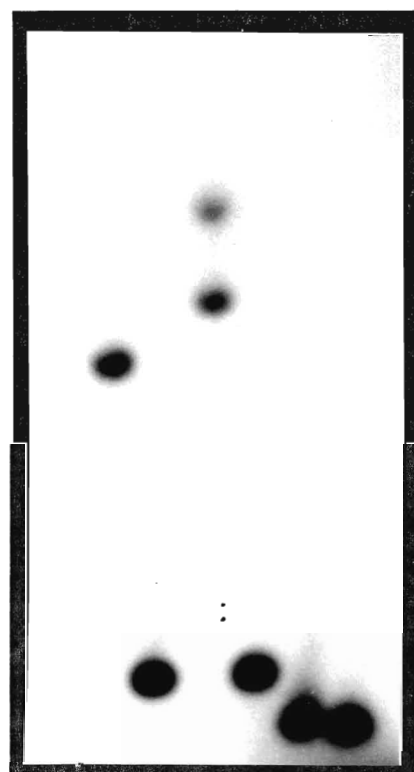
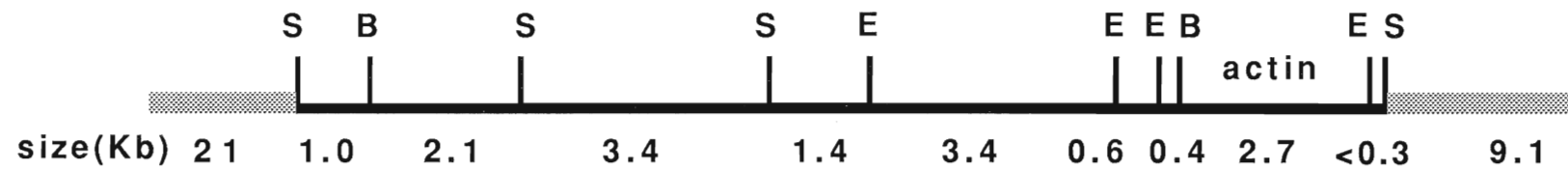


Fig.21

A restriction map of EMBL3 phage clone 16AA, containing a 15 to 16Kb newt DNA insert with homology to the chick β -actin gene. The approximate region of homology is indicated. Thick lines represent the vector arms.

EMBL3 Clone 16AA



S - SalI

E - EcoRI

B - BamHI

PLASMID CLONING

The EMBL3 phage vector is useful for the creation and screening of a genomic library, but once the actin homologous regions of these two phage clones were restriction mapped, it was necessary to subclone these sequences, as short DNA fragments, into a plasmid vector to facilitate their further characterization.(see Fig.7) The pTZ19R vector(Fig.9) was chosen for its many useful features.(see Methods)

DNA from 11A and 16AA was digested with Sall and EcoR1 respectively, and the fragments were separated by agarose gel electrophoresis. The 5.7 Kb Sall fragment from 11A and the 3.1 Kb EcoR1 fragment from 16AA were excised from the gel and purified. pTZ19R plasmid DNA was digested with either Sall or EcoR1 and then dephosphorylated to prevent religation. The 11A Sall and 16AA EcoR1 fragments were then ligated to the multicloning site of appropriately digested pTZ19R plasmid DNA.

DH5 α E. coli cells were transformed with the recombinant plasmids. White bacterial colonies, harbouring insert bearing plasmids, were selected and streaked on plates to produce single colonies. Small scale plasmid DNA preparations were made from single colonies. This DNA was restriction enzyme digested and the fragments separated by electrophoresis through agarose, to ensure that inserts were present.(Fig.22a) Colonies which did contain plasmids with inserts were restreaked, one colony per plate. Small scale preparations from these colonies were used for restriction fragment analysis to determine the insert size.

Fig.22a,b,c

Fig.22a shows an EtBr stained gel of plasmid DNA purified from transformed E. coli clones. The bacteria were transformed with ligation mixtures containing the plasmid pTZ19R ligated to either the 5.7 Kb Sall fragment from phage 11A or the 3.1 Kb EcoR1 fragment from phage 16AA. The DNA in the 11A lanes was partially digested with Sall, and that in the 16AA lanes was partially digested with EcoR1. Lanes a,b and both of the 16AA lanes show plasmids with inserts. Those lanes to the left of lane a have no inserts.

Fig.22b,c lanes a and b show restriction analyses of plasmid DNA purified from bacterial clones that were derived from the clones that produced lanes a and b in Fig.22a. The plasmids in Fig.22b were digested with EcoR1 and Sall, those in Fig.22c were digested with just Sall. The plasmid in lane a was called pTNA1. The 1.9Kb fragment in Fig.22b, lane a, results from only partial digestion of the EcoR1 site furthest to the right in the restriction map, Fig.25.

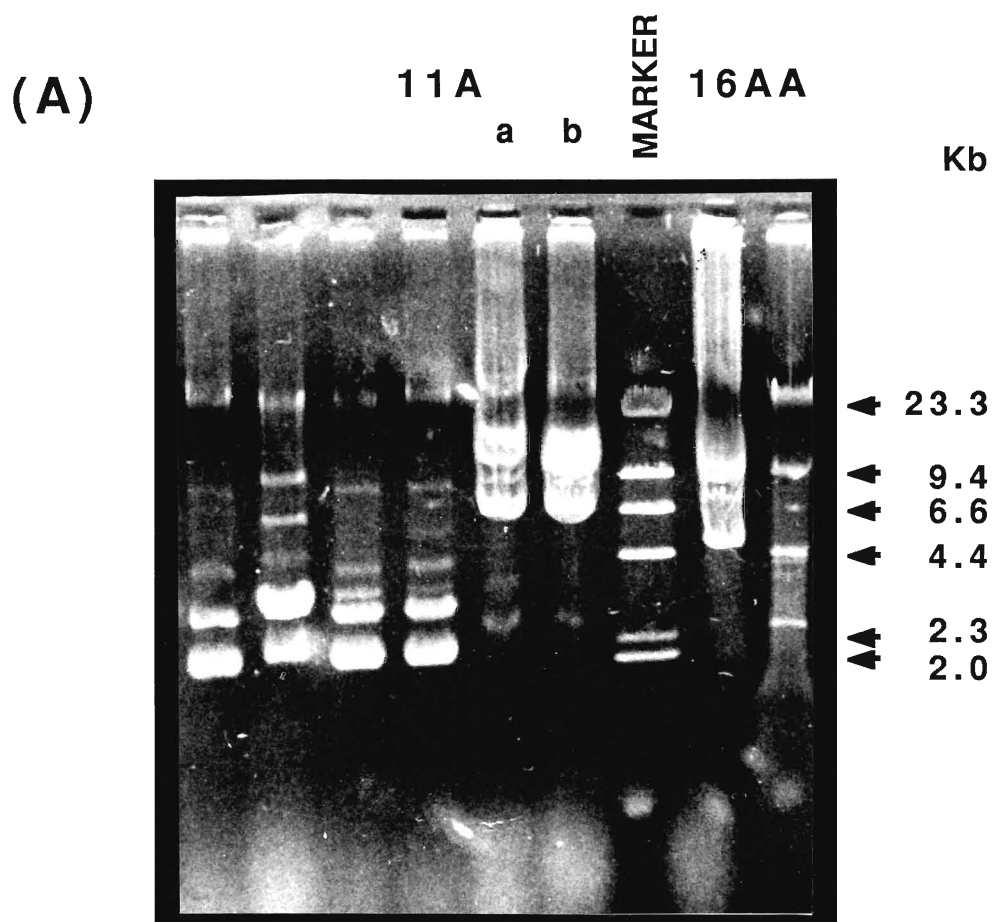
RESTRICTION MAPPING OF PLASMID CLONES

Plasmid DNA from colonies which contained 11A recombinant plasmids were digested with both EcoR1 and Sal1(Fig.22b) or just Sal1.(Fig.22c) A colony which harboured a plasmid with a 5.7kb Sal1 insert was selected. This plasmid, which contained the desired insert, was named pTNA1. Further restriction digests were made of the insert,(Fig.23,24a) resulting in the map shown in Fig.25.

Plasmid DNA from colonies which contained 16AA recombinant plasmids was digested with both BamH1.(Fig.26) This was necessary to distinguish plasmids containing the 3.1Kb EcoR1 insert from plasmids ligated to another 2.9Kb plasmid. A colony was selected which harboured a plasmid giving 5.6 and 0.4 Kb fragments indicative of the 3.1Kb EcoR1 fragment from 16AA inserted into pTZ19R with its BamH1 site closest to the Sal1 side of the multicloning site. This plasmid was designated pTNA2. Restriction digests(Fig.27a,28a) allowed a restriction map to be determined for this plasmid, as shown in Fig.29.

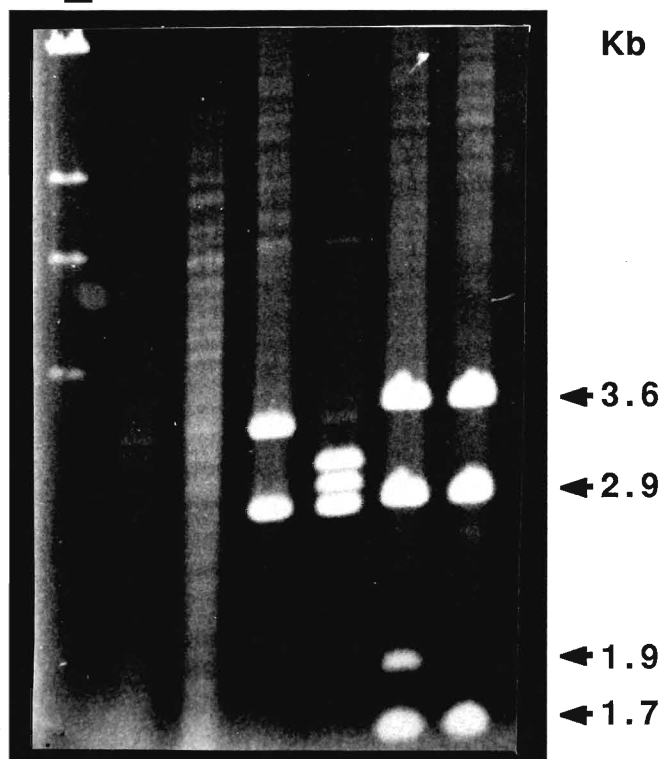
Southern analysis of pTNA2(Fig.27b,28b) indicated that the actin homology was limited to the region indicated in Fig.29. Note also that the plasmid vector sequences hybridize to the actin probe,(for example, the 2.9Kb band in Fig.28b, lane 8) but not as strongly as the insert fragments.(the lower bands in Fig.28b, lanes 4-6)

Southern analysis of pTNA1 revealed that there was virtually no hybridization to the insert,(Fig.24b,27b) but there was hybridization to the pTZ19R sequences.(for example



(B)

MARKER



(C)

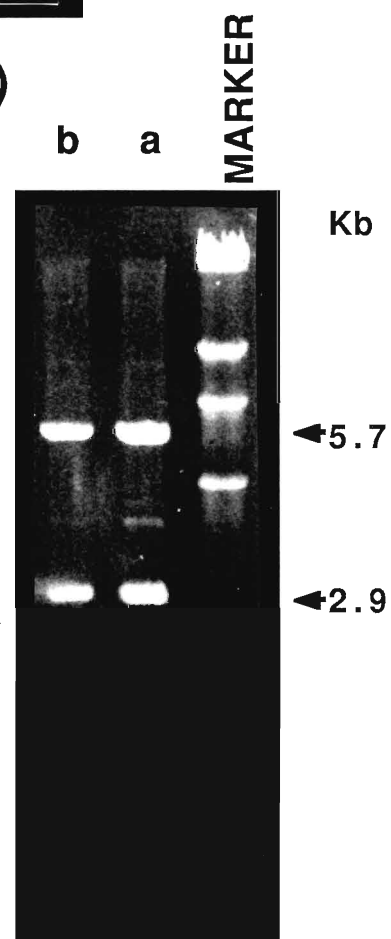
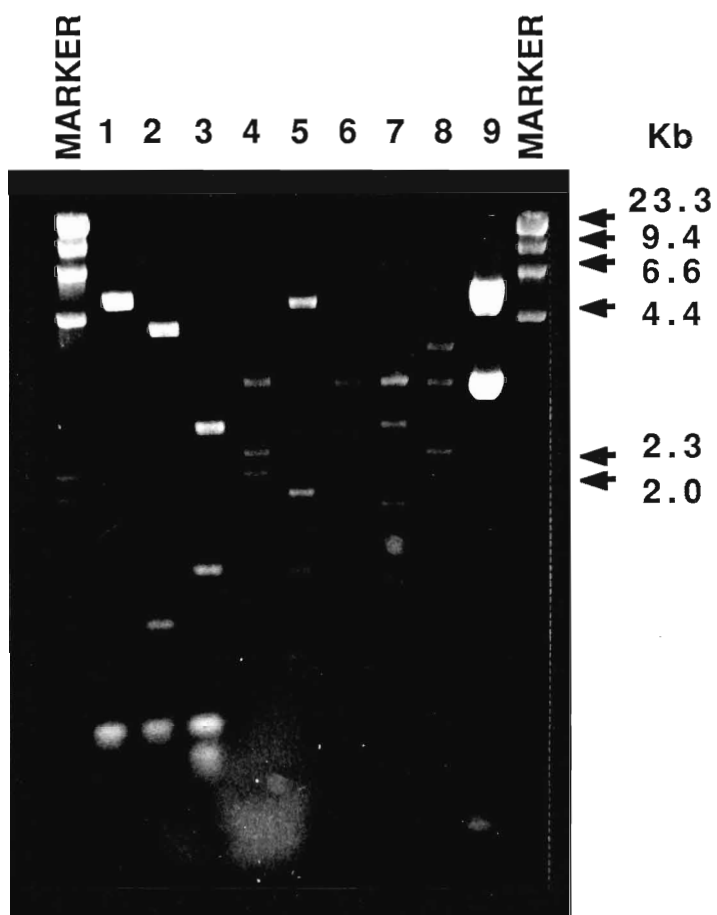


Fig.23

An EtBr stained gel showing restriction fragments of the plasmid pTNA1. The numbers above the lanes correspond to the restriction digestions listed below.



- 1 Msp1
- 2 " /HindIII
- 3 " /Pst1
- 4 Kpn1/BamH1
- 5 " /EcoR1
- 6 Pst1/BamH1
- 7 " /EcoR1
- 8 BamH1
- 9 Xho1/Sal1

Fig.24a,b

Fig.24a shows an EtBr stained gel with further restriction digestions of pTNA1. Fig.24b is the autoradiograph of a Southern blot made from the above gel and hybridized with the actin probe. Some of the fainter bands result from incomplete restriction enzyme digestions.

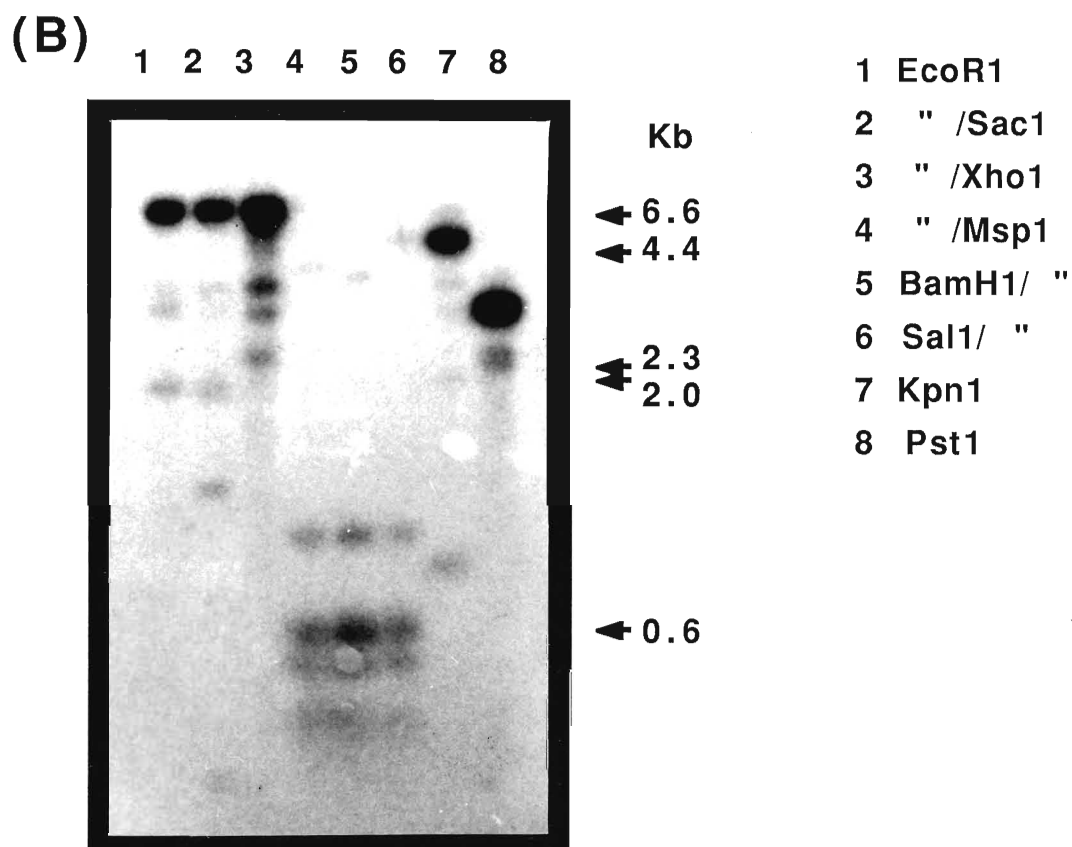
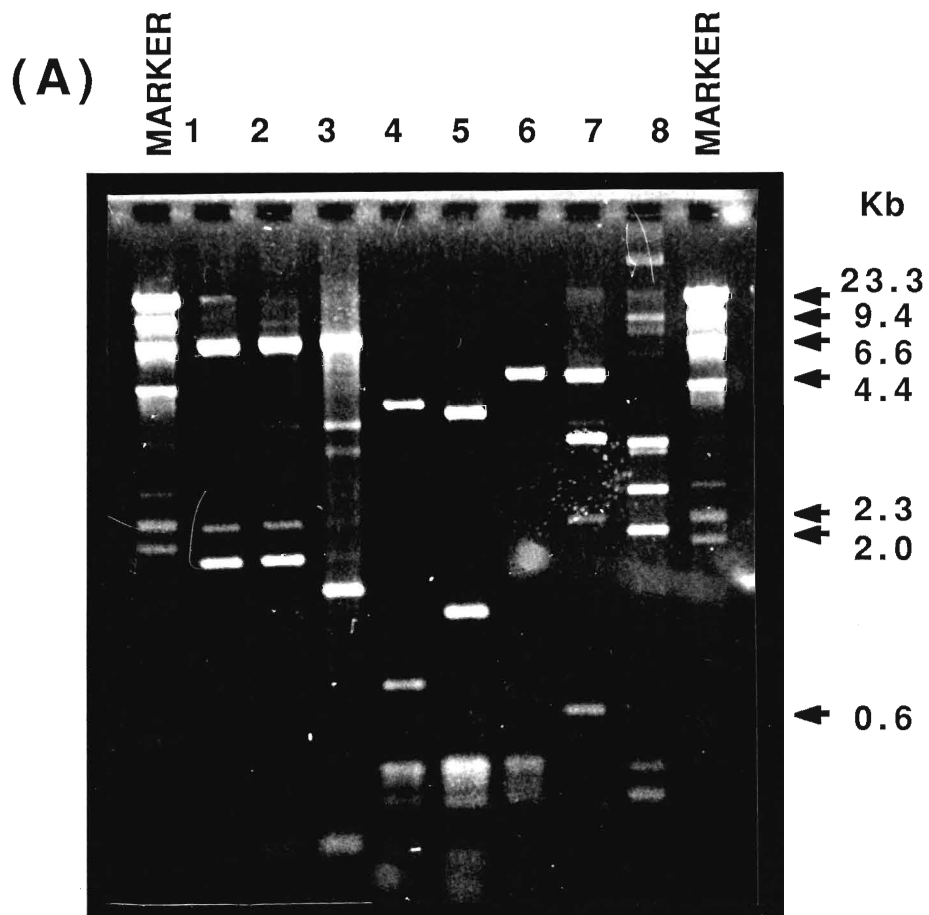
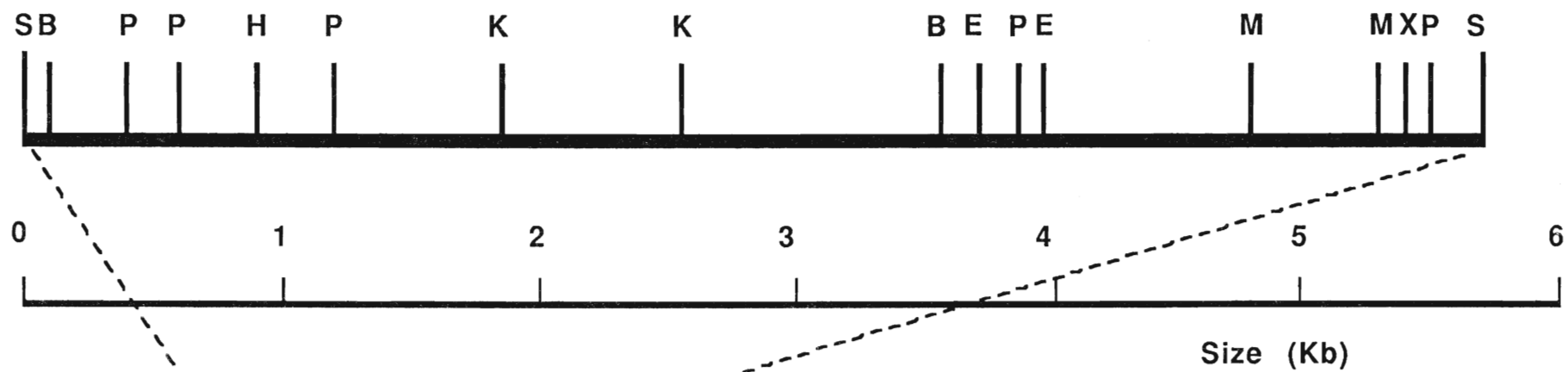


Fig.25

Restriction map of the 5.7 Kb newt DNA insert in pTNA1. The approximate region of actin homology is indicated. The newt DNA fragment was inserted into the Sall site of the puc19 multicloning site oriented as indicated. The restriction sites are: E- EcoR1, M- Msp1, B- BamH1, S- Sall, P- Pst1, H- HindIII, K- Kpn1, X- Xho1. There appeared to be no Sac1 sites within the newt insert.



pTZ19R

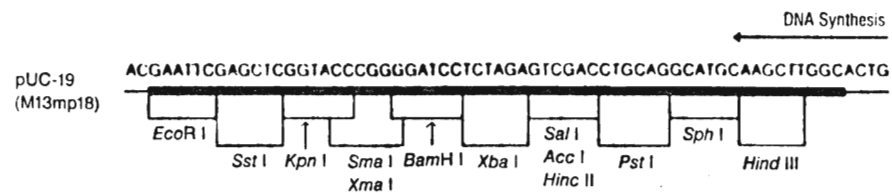


Fig.26

This EtBr stained gel shows plasmid DNA from E. coli transformed with ligation mixtures containing pTZ19R ligated to either the 0.5 or 3.1 Kb EcoR1 fragments from the phage 16AA. The plasmid DNA was partially digested with BamH1. The plasmid in lane b, containing the 3.1 Kb EcoR1 fragment from phage 16AA, was designated pTNA2. The 6.0 Kb band is the linearized plasmid. The 0.4 Kb band is the left hand BamH1 fragment shown in the restriction map Fig.29.

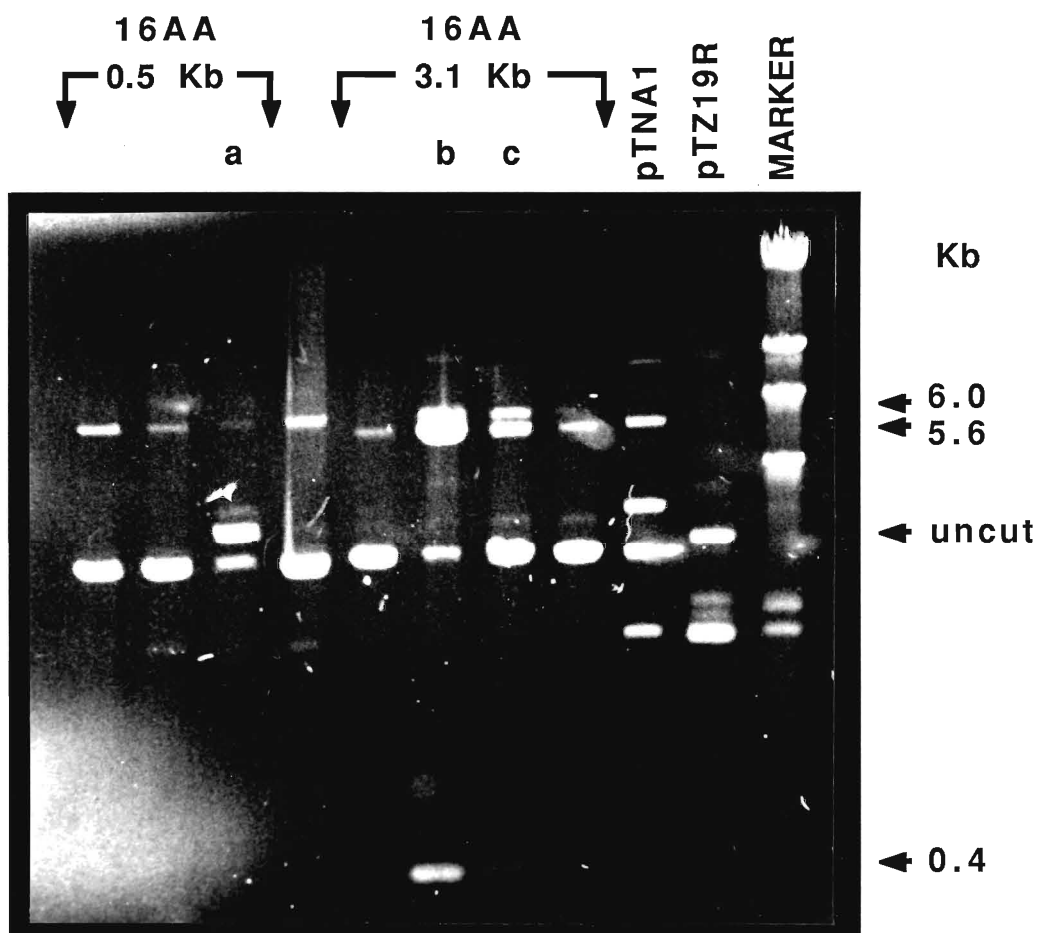
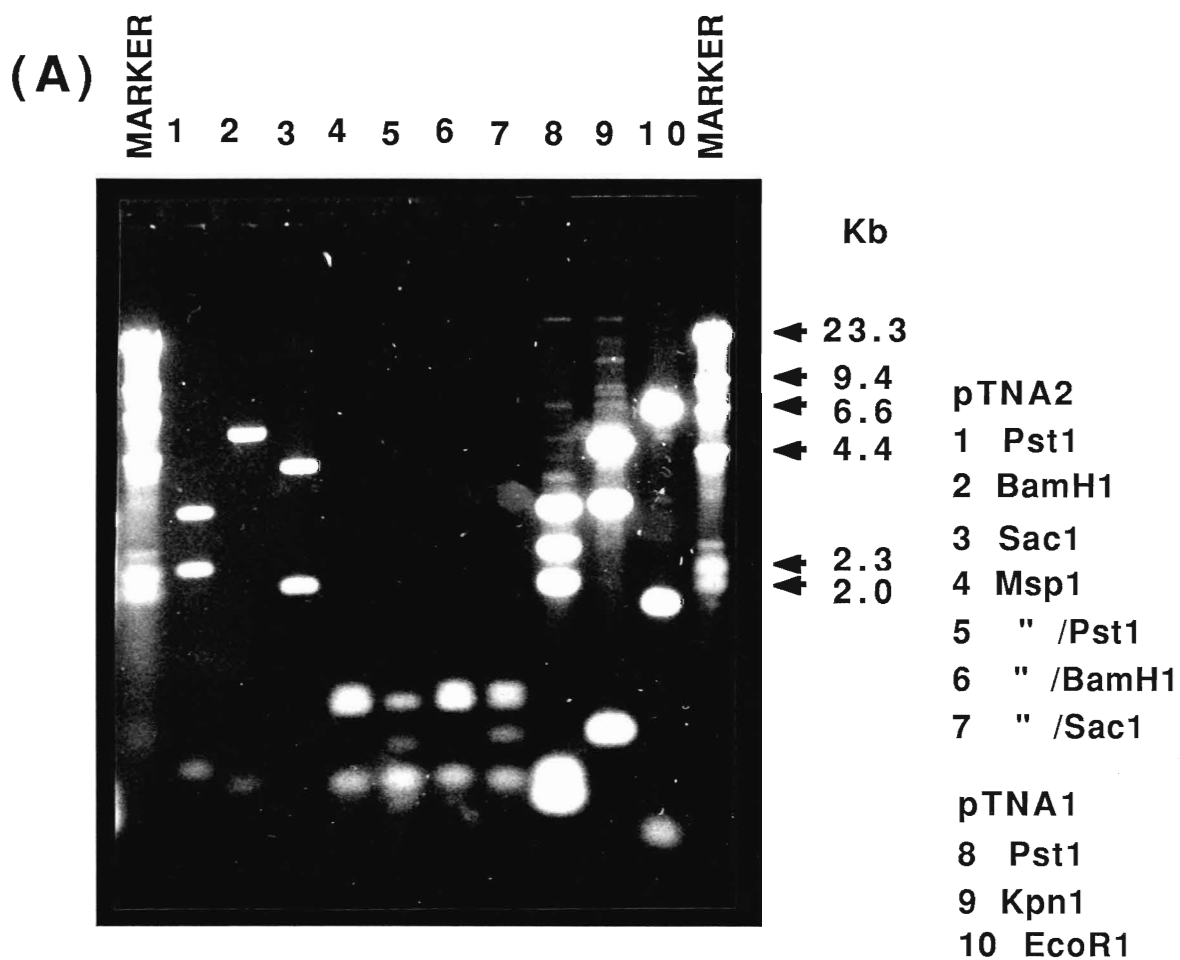


Fig.27a,b

Fig.27a shows an EtBr stained gel with restriction digestions of pTNA2 and pTNA1. Fig.27b is the autoradiograph prepared from the gel above and hybridized with the actin probe.



(B) 1 2 3 4 5 6 7 8 9 10



Fig.28a,b

Fig.28a shows an EtBr stained gel with restriction digestions of pTNA2. A 1.2% agarose gel was used to separate small fragments. The autoradiograph of the corresponding Southern blot is shown in Fig.28b.

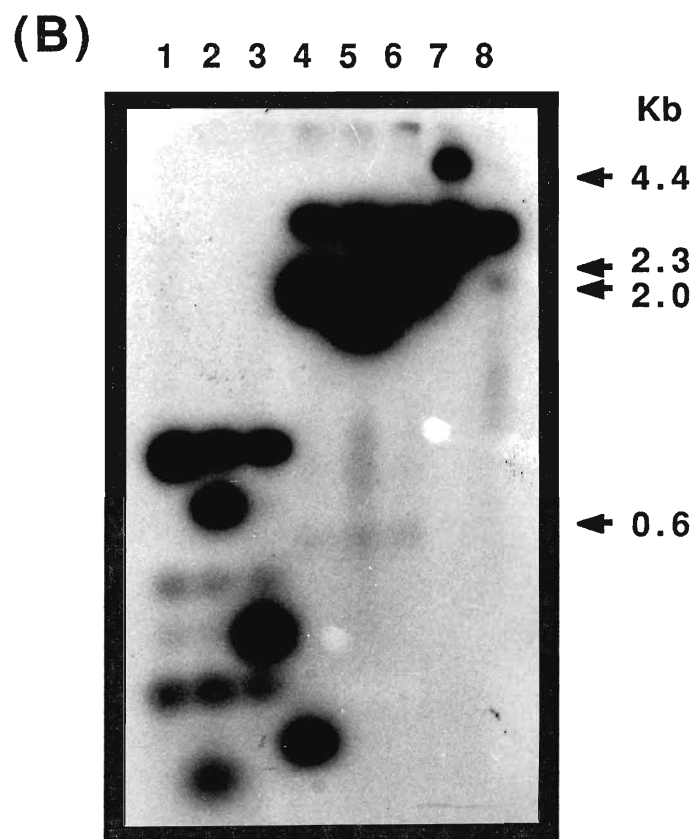
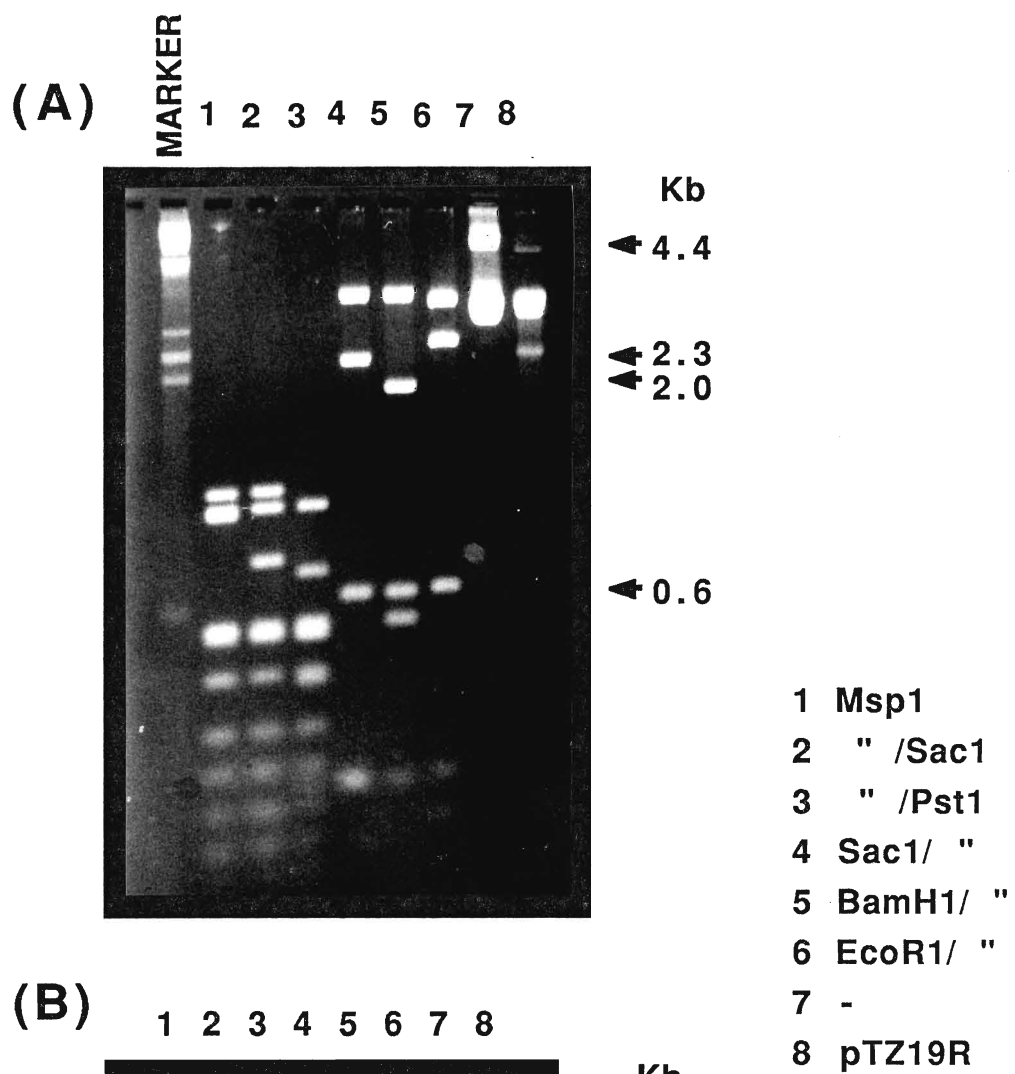
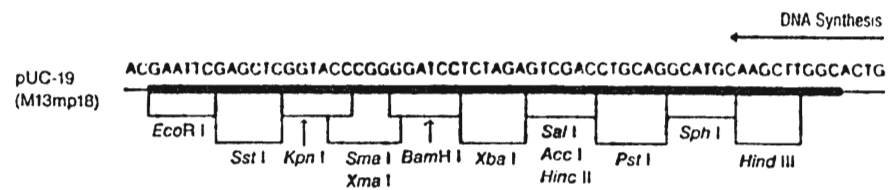
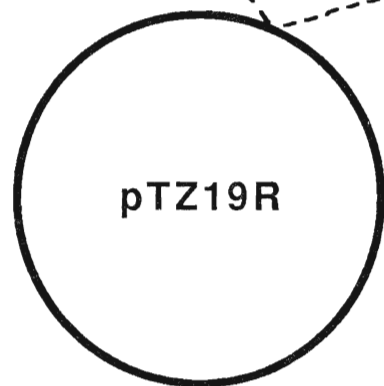
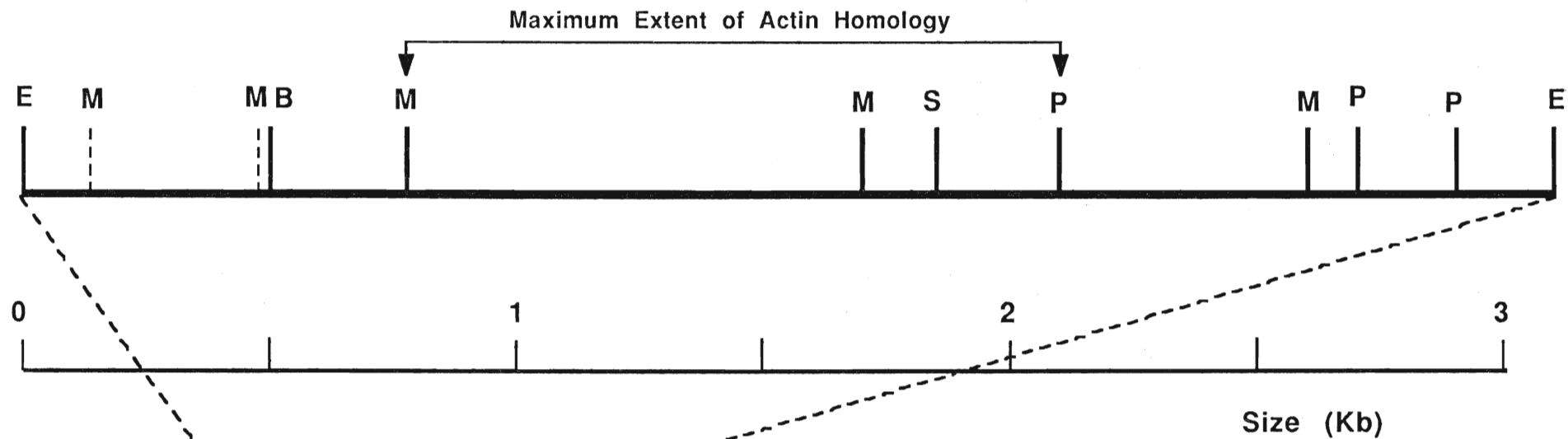


Fig.29

Restriction map of the 3.1 Kb newt DNA insert in pTNA2. The location of restriction enzyme sites with dotted lines are only approximate. The approximate region of actin homology is indicated. The newt DNA fragment was inserted into the EcoR1 site of the puc19 multicloning site oriented as indicated. The restriction sites are: E- EcoR1, M- Msp1, B- BamH1, S- Sac1, P- Pst1. The insert was not cut at any sites by Sal1, Kpn1 or Xho1.



the 3.3 Kb Pst1 band in Fig.24b, lane 8. Because of the strong hybridization of the actin probe to pTZ19R sequences, it was not possible to localize the actin homologous region within pTNA1, as explained in the discussion.

DISCUSSION:

The results of the dot blot indicated that the chick actin probe probably had more homology to sequences in the newt genome than the other two probes, although the c-fos probe appeared to have substantial homology with newt sequences. The c-myc probe did not have any detectable homology even at this low stringency.

The major problem with dot blots is that non-specific hybridization would appear the same as genuine sequence specific hybridization. In this case though, several characteristics of the results lend confidence to the interpretation above. The same blot was hybridized with three different probes, and each probe gave a different pattern of hybridization. The mouse probes hybridized best to mammalian DNA, but the chick probe hybridized best to chick DNA. The actin gene probe which is very highly conserved evolutionarily and is present in many copies in the genomes of all vertebrates studied,^{43,48} hybridized better to the newt DNA than did the c-fos or c-myc probes.

Southern blots have the advantage over dot blots of separating the DNA by size, thereby allowing one to distinguish the banding patterns of sequence specific hybridization from nonspecific binding to the DNA.

Several of the results of the first Southern were unexpected. Although both c-fos and actin hybridized to the newt DNA on the dot blot, no bands were detected in the newt

lane of the Southern with either of these probes. This is particularly unusual since the actin probe identified the single yeast actin gene which is likely to be substantially more divergent from the probe than newt actin genes.

There are several explanations for these results. A major factor is that there were 15 times fewer newt genomes applied to the Southern blot than with the other species, due to the huge size of the newt genome. Also, the smear in the newt lane, rather than distinct bands, suggests that the DNA was incompletely digested or partially degraded. This is also evident with the mouse DNA.

The problem with identification of newt genes using Southern analysis was emphasized by the results of the two other Southern blots. Fig.12 shows that the chick actin probe was able to identify actin genes in a variety of widely divergent species, but not newt. Another Southern blot was prepared with newt DNA digested by 7 different restriction enzymes, but the actin probe did not identify any distinct bands, just smears.

We decided to screen the library for the actin gene for the following reasons. 1. The actin probe hybridized to newt DNA on the dot blot to a greater extent than either the c-fos or c-myc probes. 2. The actin gene is very well conserved evolutionarily.⁴⁸ The fact that the actin probe identified the yeast actin gene indicated that, at the low stringency used, there was enough evolutionary sequence conservation for the chick actin probe to hybridize to actin genes from very distantly related species. 3. The large size of the

cytoplasmic actin gene family greatly increased the chance of cloning one of its members.

It was surprising that the actin probe detected such strongly hybridizing bands when DNA from the newt genomic library was analyzed by Southern blot. Two factors that might have contributed to this were, that library DNA was more easily purified without degradation or shearing, and was more easily digested with restriction enzymes than newt genomic DNA. In any case, the results of the EMBL Southern indicated that isolation of a newt actin gene from the library was feasible.

The primary library screening produced one to two positive plaques per plate. This gives an estimate of 200-400 β -actin related sequences per haploid genome (calculations in Appendix I). This is about ten times the number found in mammals. If it is accurate, this would substantiate Minty's observation,⁴¹ that the number of genes in the actin family is roughly proportional to the genome size, since the newt genome is ten times as large as the mammalian genome.

The later primary screening that produced clone 16AA gave contradictory results. Only 20,000 pfu were plated, but at least ten strongly hybridizing plaques were present, and many weaker signals. This frequency is 2 to 5 times that of the first screening. A possible explanation is that the improvements in the hybridization procedure, such as the use of a higher specific activity probe, allowed the detection of sequences which had less homology to the chick actin probe

than in the primary screening.

When first attempted, secondary screening of the library was plagued by a high level of background on the hybridization membranes. Problems with non-specific background could have been caused by several factors. There was difficulty obtaining consistently labelled probe using the nick translation procedure. Probe lengths which are too long or too short can cause high background.⁸² The length of the probe also affects the stability of its hybridization to the DNA on the blot.⁸³ With nick translation the probe length can vary widely according to a number of factors.⁸² Not only did the random primer technique give a higher specific activity probe, but it gave results that were more reproducible. Use of the actin gene fragment, rather than the actin gene inserted in a plasmid, also increased the specific activity of the probe. Only sequences that should hybridize to the target gene sequence are labelled, so there is no extra radioactivity added with the probe which could contribute to an increase in the overall background.

Another source of high background could have been the use of dextran sulfate in the hybridization solution, which has been reported to increase background.^{73,83} It is routinely used by many investigators, however, to decrease hybridization time and give a better signal to noise ratio. Although I had used it with no problems for the previous Southern and dot blots, background seemed significantly reduced with its removal from the hybridization solution in subsequent procedures.

Although, the background problems made secondary screening of the 20 primary plaques isolated from the library less reliable, it would not have contributed to the apparent loss of actin homologous DNA from the replated plaques or particularly from the Southern blot of DNA purified from the primary plaques. The most likely explanation, supported by the work of others,^{16,25} is that EMBL3 clones, including those homologous to the chick actin gene, were lost as the phage were propagated.

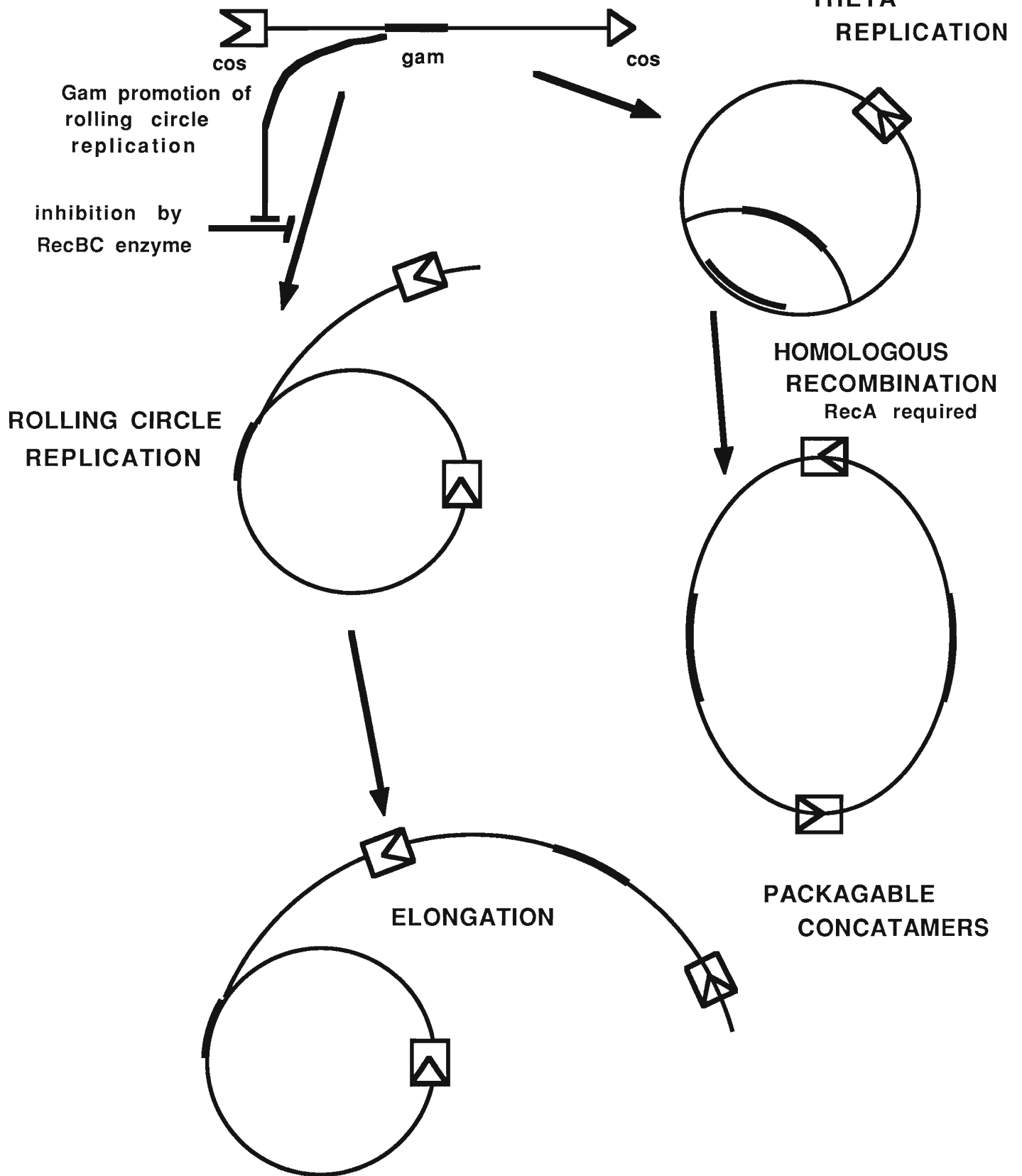
The replication of EMBL3 phage is illustrated in Fig.30. Propagation of the phage is dependent on production of concatenated molecules of lambda DNA which can be packaged. The predominant mode of replication for wild type lambda phage is by rolling circle. When EMBL3 is propagated with the most commonly used *rec+* bacterial strains as hosts, rolling circle replication is blocked by the host RecBC enzyme since recombinant EMBL3 phage lack the lambda *gam* gene. Concatenated phage DNA can only be derived by homologous recombination of closed circular DNA produced by theta replication. This requires the host RecA recombination enzyme and is stimulated by the *chiD* sequence in the right arm of EMBL3.²⁵ Unfortunately this active recombination of phage DNA presents a serious problem when working with a new library, particularly when screening for the actin gene family.

Inserts in EMBL3 which contain repeated DNA may be lost due to inefficient propagation as recombination takes place at sites within the insert, particularly when limited to

Fig.30

EMBL3 phage replication. Some of the E. coli recombination(Rec) enzymes that influence replication of the phage are indicated.

LAMBDA PHAGE



theta replication. Newt DNA contains a high level of uniformly dispersed repeated sequences, which could destabilize many of the recombinant phage. Furthermore most of the actin genes are flanked by repetitive DNA.^{22,30,48}

Muller et al¹⁶ attempted to clone genes from a Pleurodeles waltl genomic library. This urodele has a genome which is similar in size to that of N. viridescens, and also contains many highly repetitive sequences. They reported that the cloning efficiency was 10 to 100 times less efficient with P. waltl DNA than for other vertebrates. Several observations led them to attribute this to loss of phage during propagation due to recombination of repetitive DNA in the inserts. Muller acceded to "enormous problems" in cloning large, highly repetitive genomes.¹⁶ Wyman and Wartman have reviewed the problem of cloning repetitive DNA.²⁵ To reduce recombination, they suggest the use of a bacterial host, deficient in the recombination genes, *recA* and/or *recB, recC, sbcB*. In particular, the *recB-, recC-* mutation allows the more prolific rolling circle replication mode. (The *sbcB* mutation suppresses deleterious effects of the former mutations.)

When such a host, MB406, was used for propagation of phage from the EMBL3 newt library, secondary screening was successful: as described in the results section, replating of primary plaques using MB406 gave many positive plaques.

Two phage clones 11A and 16AA, which hybridized to the actin probe, were isolated from the library and determined to have completely different restriction maps. This indicated

that they were independent actin homologous sequences. Further restriction mapping of the fragments cloned into pTZ19R supported this conclusion, as there were no restriction site patterns in common. The shorter autoradiographic exposure time necessary for the Southern blots of 16AA(two hours), compared to the Southern blots of 11A(20 hours), may indicate that the newt sequence in 16AA is more closely related to the chick β -actin gene than the sequence in 11A.

Southern analysis of plasmid pTNA2 localized the actin homologous sequences to the region indicated in Fig.29. An estimate of the maximum extent of the actin homologous region is about 1.3 Kb. This is too short to be one of the functional cytoplasmic actin genes, which are greater than 3 Kb, therefore the sequence may represent a 'reverse transcript pseudogene'. In favour of this possibility, is the fact that pseudogenes outnumber functional genes by 20-50 to one in other vertebrates.⁴³

Southern analysis of the plasmid pTNA1 revealed two problems. The chick actin probe cross-reacted with the plasmid vector pTZ19R, but did not appear to hybridize to the pTNA1 insert. The most likely explanation for hybridization of the actin probe with pTZ19R is that during purification of the chick actin fragment from the plasmid, some pGEM sequences contaminated the preparation. Since the uncut plasmid containing the chick actin gene has the same mobility in agarose gels as the 2.0 Kb actin cDNA fragment, this may have escaped detection. pGEM and pTZ19R are derived from the

same plasmid and are identical throughout most of their sequence, therefore the labelled pGEM sequences contaminating the probe would bind very strongly to any pTZ19R sequences on the Southern blots. This didn't cause a problem when screening the library because EMBL3 does not contain sequences with homology to pGEM.

The lack of hybridization of the chick actin probe to the pTNA1 insert seems more perplexing. The insert had the same size and restriction sites as the 5.7 Kb SalI fragment of the phage clone 11A, in which actin homologous sequences were clearly detected. The likely explanation is that pGEM DNA, contaminating the probe, hybridized so strongly to pTZ19R DNA on the Southern blots that it gave dark bands after only a short exposier to X-ray film. The more weakly hybridizing actin sequences would have required an exposier ten times this, as reported for Southern blots of phage 11A, and therefore were relatively very faint. There was no such problem with pTNA2 because the newt sequence within it hybridized even better to the actin probe than contaminating pGEM did to pTZ19R.

In order to solve this problem, the actin fragment would have to be more carefully purified from pGEM1 and tested for absence of hybridization with pGEM1 DNA. Southern analysis of pTNA1 could then be repeated.

Cloning of newt actin gene family members makes possible studies investigating the genomic organization of the newt actin gene family as well as the expression of actin genes during limb regeneration. The insert in pTNA2 should be

sequenced to determine its relationship to other members of the cytoplasmic actin gene family. I have been unable to find any published sequences of urodele actin genes. The actin isotype could be determined using the N-terminal acidic amino acid classification system of Vandekerckhove.²⁹ Sequence comparison with previously cloned actin genes from other species would give some information about the evolutionary relationship of the newt actin gene to other actin genes. Using the pTNA2 insert as a probe would facilitate the cloning of other members of the newt actin gene family.

With the pTNA2 insert as a probe, expression of cytoplasmic actin genes during limb regeneration could be investigated, using in situ hybridization to tissue sections. It would be of interest to know whether expression varied spatially, temporally or changed in response to experimental manipulations such as denervation or retinoic acid treatment. Investigations such as these will lead to a better understanding of the processes of cellular proliferation and differentiation which occur during limb regeneration.

SUMMARY:

1. Molecular cloning of newt sequences from a genomic library seemed to be hampered by loss of EMBL3 phage during propagation. This is likely caused by recombination within the newt DNA due to the large number of repetitive DNA sequences, as reported by others. Use of a *recB*⁻, *recC*⁻ host, MB406, apparently alleviated this problem.

2. Two separate screenings of an *N. viridescens* genomic library produced β -actin homologous clones at estimated frequencies of 200-400 copies/genome and 1000-2000 copies/genome. The latter estimate is probably more accurate.

3. Restriction maps were produced for two recombinant phage, 11A and 16AA, which contained actin homologous newt sequences within their 15 to 17 Kb inserts.

4. 5.7Kb and 3.1Kb fragments from 11A and 16AA respectively, containing the actin homologous sequences, were subcloned into the pTZ19R plasmid vector, producing plasmids designated pTNA1 and pTNA2. Detailed restriction maps were produced for the inserts of these plasmids.

5. The short(< 1.3 Kb) length of the actin homologous newt sequence in pTNA2 indicated that it was possibly a cytoplasmic actin processed pseudogene.

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APPENDIX I:

a/ Calculation of the number of clones needed for a complete genomic library.

Using the formula from Maniatis:⁷³

$$N = \frac{\ln(1 - p)}{\ln(1 - f)}$$

p = desired probability
f = fraction of the genome in a single recombinant phage
N = number of phage to screen

- for N. viridescens, EMBL3 genomic library

$$\begin{aligned} \text{genomic size} &= 45 \text{ pg/haploid} \times 0.966 \times 10^9 \text{ bp/pg}^{19} \\ &= 4.3 \times 10^{10} \text{ bp/genome} \end{aligned}$$

$$\text{therefore } f = \frac{20 \times 10^3 \text{ bp/phage}}{4.3 \times 10^{10} \text{ bp/genome}}$$

probability = 95%

$$N = \frac{\ln(1 - 0.95)}{\ln(1 - 4.6 \times 10^{-7})}$$

= 6.4×10^6 phage must be screened for a 95% chance of finding a single copy gene.

b/ Calculation of the actin gene copy number in the newt genome.

$$\frac{\text{actin genes}}{\text{haploid genome}} =$$

$$= \frac{\begin{matrix} 1 \text{ to } 2 \\ \text{positive plaques} \end{matrix}}{10^4 \text{ phage/plate}} \cdot \frac{20 \times 10^3 \text{ bp}}{\text{phage}} \cdot \frac{4.3 \times 10^{10} \text{ bp}}{\text{genome}}$$

= 200 to 400 copies of actin in the genome

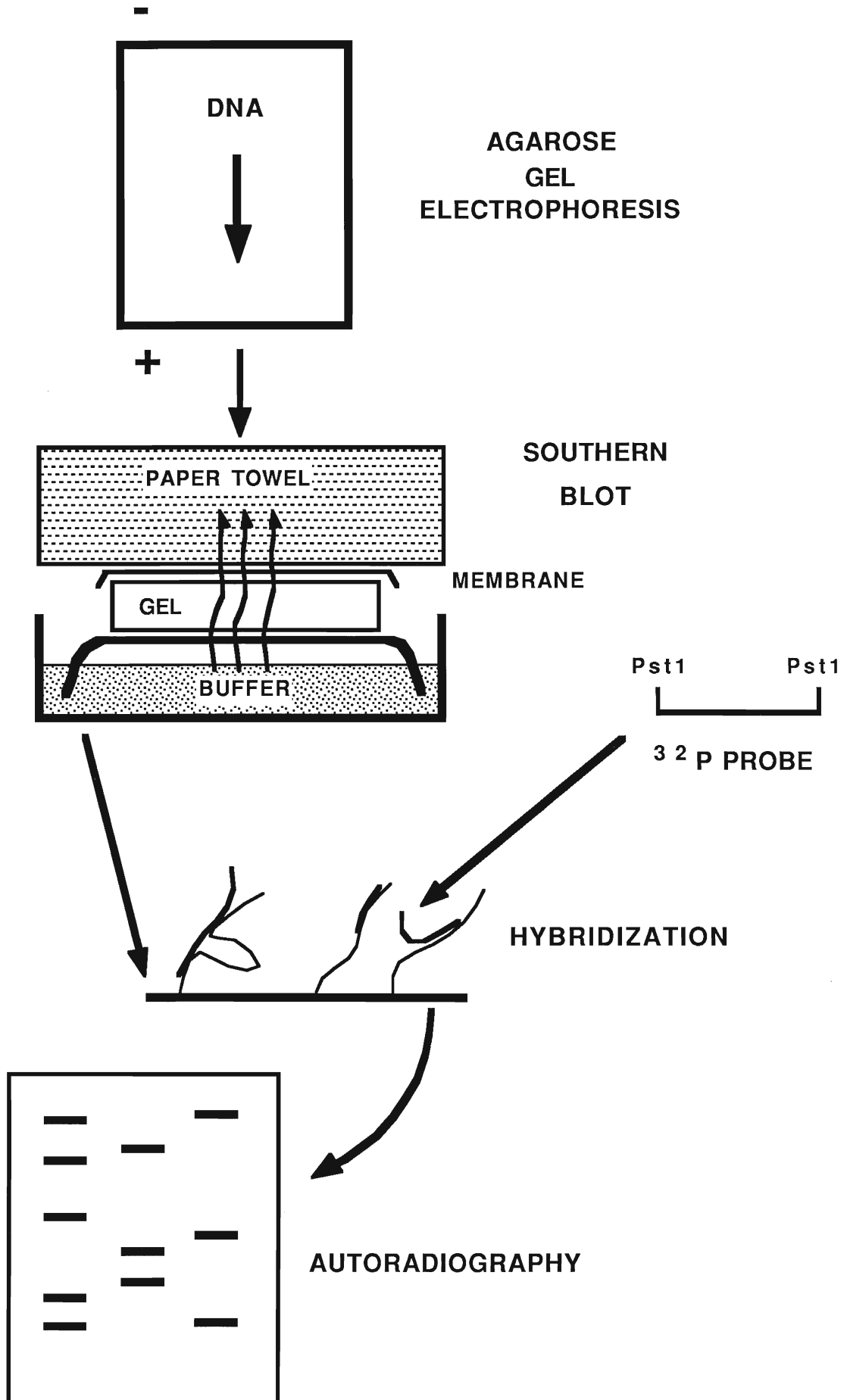
- For ten positive plaques in 20,000 pfu the estimate would be up to 2000 actin genes.

APPENDIX II:

Diagram of the Southern Blot protocol.

Fig.31

Southern blot protocol. DNA fragments produced by restriction digestion are separated by size using gel electrophoresis. The DNA is then denatured and transferred to a DNA binding membrane by the capillary action of high salt buffer flowing through the gel and membrane into a stack of paper towels. Once bound to the membrane the DNA can be hybridized to single-stranded, radioactive DNA sequences(thick lines). After washing, the radioactive probe sequences remain hybridized to homologous sequences tethered to the membrane, and can be visualized by exposure to X-ray film.



APPENDIX III:

Factors that affect the kinetics and stringency of DNA hybridizations.

a. Hybrid stability varies according to the following equation from Wahl⁸³:

$$T_m = 81.5 + 0.41(G + C) + 16.6 \log[Na^+] - 0.62(\% \text{ formamide}) - 500/L$$

- T_m - melting temperature of the duplex in °C.
- $(G + C)$ - percentage of guanine and cytosine in the DNA.
- $[Na^+]$ - the molarity of Na^+ or equivalent monovalent cations in the solution.
- L - the average probe length in nucleotides.
- formamide - an agent used to reduce the stability of DNA duplexes, allowing hybridizations to be done at lower temperatures
- the T_m is lowered 1°C for every 1% mismatch between the probe and target sequences.⁸³

In general the stringency of hybridization is usually controlled by the post-hybridization washing conditions. Hybridizations are carried out at ($T_m - 25^\circ\text{C}$), which is a low stringency and approximately the temperature of maximum renaturation rate. Washing can then be carried out at higher stringency, (0.33M Na^+ , 65°C) the degree of stringency being dependent on the amount of probe/target sequence mismatching.⁸³

b. The rate of hybridization using a double-stranded DNA probe approximately follows the equation from Wahl⁸³:

$$t_{1/2} = \frac{(F)(N)(ml)}{(\mu g \text{ DNA})(10000)}$$

$t_{1/2}$ - the number of hours for half of the probe to renature.

F - the ratio of the rate of the hybridization under the conditions used to the rate of the hybridization under standard conditions. (Standard conditions refer to a solution temperature of ($T_m - 25^\circ\text{C}$), 0.18M cation concentration, with no formamide)

- formamide reduces the hybridization rate by 2 fold.⁸³

- dextran sulfate increases hybridization rate by up to 100 fold.⁸³

- hybridizations proceed 7 times faster in 1M Na^+ concentrations than they do in 0.18M Na^+ .⁸³

N - the sequence complexity of the probe. (the length of a nonrepetitive probe in bp)

ml - volume(ml) of the hybridization solution

$\mu\text{g DNA}$ - amount of probe added

Hybridization reactions are usually carried out for 3 X $t_{1/2}$, when they are considered essentially complete. In this project, hybridizations included 40 to 100ng of a 2Kb probe, 50% formamide, 1M Na^+ , in a 10ml solution. Therefore F is 2/7, the hybridization time was calculated as follows.

$$t_{1/2} = \frac{(2/7)(2000)(10)}{(0.04)(10000)}$$

$$= 14 \text{ hrs}$$

- therefore hybridizations were carried out for 48 hrs.

- when dextran sulphate was present, hybridizations were carried out overnight.